Nutrient Modification of the Innate Immune Response

A Novel Mechanism by Which Saturated Fatty Acids Greatly Amplify Monocyte Inflammation

Eric A. Schwartz; Wei-Yang Zhang; Sheetal K. Karnik; Sabine Borwege; Vijay R. Anand; Phyllis S. Laine; Yali Su; Peter D. Reaven

Objective—Monocyte/macrophage inflammation is an important contributor to diabetes and cardiovascular disease. Studies have suggested saturated fatty acids (SFA) induce monocyte inflammation in a Toll-like receptor-4–dependent manner, but recent data suggest SFA do not directly interact with Toll-like receptor-4. The present study tests the novel hypothesis that metabolism of SFA cooperatively amplifies Toll-like receptor-4–mediated inflammation.

Methods and Results—THP-1 monocytes exposed to 100 μmol/L SFA in vitro for 16 hours followed by 1 ng/mL lipopolysaccharide demonstrated enhanced IL-6 and IL-8 mRNA and protein expression (≥3-fold higher than the sum of individual responses to SFA and lipopolysaccharide). SFA had similar effects on THP-1 macrophages and primary human monocytes. This amplified lipopolysaccharide response could be blocked by inhibition of SFA metabolism to ceramide and restored by cell-permeable ceramide. Both SFA and ceramide activated PKC-ζ and the mitogen-activated protein kinases Erk, JNK, and p38. Inhibition of these pathways prevented the SFA-induced increase in cytokine expression.

Conclusions—These results provide evidence for potent amplification of monocyte/macrophage innate immune responses by a novel pathway requiring metabolism of SFA to ceramide and activation of PKC-ζ/mitogen-activated protein kinases. These findings demonstrate how nutrient excess may modulate innate immune system activation and possibly contribute to development of diabetes and cardiovascular disease. (Arterioscler Thromb Vasc Biol. 2010;30:00-00.)

Key Words: ceramide • mitogen-activated protein kinase C • monocytes • protein kinase • Toll-like receptor
TLR also recognize numerous endogenous molecules. Studies by Hwang, Flier, and others\textsuperscript{14–16} have suggested that elevated concentrations of saturated fatty acids (SFA) lead to activation of TLR2 and TLR4. Because Western diets are typically enriched in SFA, excess nutritional intake may increase plasma and tissue SFA concentrations, potentially inducing inflammation and contributing to development of insulin resistance.\textsuperscript{17} Because reduced insulin action increases adipose tissue lipolysis and hepatic production of triglyceride-rich lipoproteins, this may further increase concentrations of plasma free fatty acids (FFA), including SFA. Therefore, elevated plasma concentrations of SFA may facilitate the initiation and progression of insulin resistance\textsuperscript{18–20} and, through this mechanism, as well as direct vascular inflammation, contribute to development of diabetes and cardiovascular disease.\textsuperscript{21}

We have previously demonstrated direct proinflammatory effects of mixtures of FFA, as well as physiologically relevant concentrations of SFA, on human monocytes and macrophages in vitro, consistent with low-level activation of TLR by SFA.\textsuperscript{22,23} However, recent studies have called into question the ability of TLR to directly bind SFA as a ligand and suggest that SFA might modulate TLR activity via lipid raft and suggest that SFA might modulate TLR activity via lipid raft.\textsuperscript{22,23} We now describe the novel finding that SFA greatly amplify the proinflammatory cytokine responses of human monocytes and macrophages to low, physiologically relevant concentrations of LPS through a mechanism separate from, but parallel to, the TLR4 signaling pathway.

### Results

Compared to control cells, THP-1 monocytes exposed to 1 ng/mL LPS for 4 hours exhibited a 21-fold increase in IL-6 mRNA expression and a 10-fold increase in IL-8 mRNA expression (Figure 1A). Exposure of these cells to 100 \( \mu \text{mol/L} \) palmitic acid (PA) for 16 hours induced a 7-fold and 2-fold increase in mRNA for these 2 cytokines, respectively. However, exposure of the cells to PA followed by LPS resulted in an 80-fold increase in IL-6 and a 53-fold increase in IL-8 mRNA expression, compared to controls. These results were significantly (\( P<0.05 \)) higher than the sum of the separate effects of PA and LPS. Confirmatory experiments with other saturated (Figure 1A) and unsaturated fatty acids, and with both macrophages and primary human monocytes, are described elsewhere (see Supplementary Results and Supplementary Figures I, II, available at http://atvb.ahajournals.org). Similar to LPS, PA-induced amplification of the monocyte response to the TLR2 ligand Pam3CSK4 was also observed (Figure III).

Exposure of monocytes to SFA also enhanced LPS-induced secretion of cytokine proteins. Compared to controls, monocytes exposed to PA for 16 hours demonstrated increased IL-6 protein secretion over the subsequent 48 hours (Figure 1B). Exposure of monocytes to 1 ng/mL LPS alone for 48 hours did not increase IL-6 protein secretion. In contrast, exposure of monocytes to PA followed by LPS increased IL-6 protein secretion by nearly 4-fold; similar fold increases were observed for IL-8. These protein changes confirm the existence of the synergistic increase in inflammation that was suggested by mRNA responses. Although there was a detectable increase in IL-6 and IL-8 mRNA in response to 4 hours of LPS treatment alone, protein secretion in response to 48 hours of LPS alone was not different from controls. In contrast, pretreatment with PA induced a
prolonged and detectable increase in LPS-stimulated IL-6 and IL-8 protein secretion.

To elucidate the underlying mechanism by which SFA amplifies monocyte inflammation, we evaluated and excluded several possibilities, including upregulation of TLR expression or increased binding affinity of Toll-like receptors, or differentiation of monocytes to macrophages (Supplementary Results and Figures IV, V). However, experiments demonstrated that at least 8 to 16 hours of exposure to THP-1 monocytes to SFA was required before the treatment amplified responses to LPS (data not shown). To test whether this time lag resulted from metabolic processing of PA by the monocytes, we examined the requirement for incorporation of PA into cells. The fatty acyl CoA synthetase inhibitor Triacin-C significantly reduced the effect of PA on the LPS response (Figure 2A). We next assessed whether conversion of PA to its metabolic product ceramide, an important proinflammatory molecule implicated in the pathogenesis of insulin resistance,26,27 played a role in PA-mediated amplification of inflammation. As shown in Figure 2B, exposure of THP-1 monocytes to 100 μmol/L PA for 16 hours significantly increased cellular C16 ceramide (the direct metabolic product of PA and the predominant ceramide in the monocytes). C18 ceramide content also increased significantly, although the increase was smaller. Furthermore, Triacin-C also blocked PA-induced increases in C16 and C18 ceramide (Figure 2B), consistent with its inhibition of the amplified LPS response. Exposure of THP-1 monocytes to 50 μmol/L C2 ceramide (a cell-permeable analogue of PA-derived C16 ceramide) similarly amplified LPS responses (Figure 2C). In contrast, the cell-impermeable C2 ceramide precursor C2 dihydroceramide did not amplify subsequent LPS responses.

Inhibitors of different enzymatic steps in the conversion of PA to C16 ceramide (specifically, the serine palmitoyl transferase inhibitor Myricin; the ketosphinganine synthetase inhibitor L-cycloserine; and the ceramide synthase inhibitor Fumonisin-B1) also effectively inhibited the ability of PA to enhance LPS-induced IL-6 and IL-8 expression (Figure 2A). Importantly, using C2 ceramide, but not C2 dihydroceramide, to bypass Triacin-C restored the amplified responses to LPS (Figure 2D), demonstrating that Triacin-C was not inducing nonspecific suppression of inflammation. Essentially identical results were observed when C2 ceramide was also used to bypass the other inhibitors of ceramide formation (data not shown). These data provide considerable evidence that generation of ceramide from SFA is required for SFA-induced amplification of LPS response by PA is ceramide-dependent. A, Monocytes were exposed to media alone (C), to media containing 100 μmol/L PA, to 1 ng/ml LPS, or to 100 μmol/L PA followed by LPS (PA LPS) in the absence or presence of inhibitors of de novo ceramide synthesis (100 μmol/L Triacin C [TriC]; 1 μmol/L Myricin [Myr]; 250 μmol/L L-cycloserine [LCS]; and 10 μmol/L Fumonisin B1 [FB1]). IL-6 (white bars) and IL-8 (black bars) mRNA was measured by real-time polymerase chain reaction. B, Cells were exposed to media or PA in the absence (PA) or presence (PA TriC) for 16 hours. C16 (black bars) and C18 (white bars) ceramide concentrations were measured by mass spectrometry. C, Cells were exposed to media, LPS, C2 ceramide (C2), C2 dihydroceramide (diC2), or C2 or diC2 followed by LPS (C2 LPS or diC2 LPS). IL-6 and IL-8 mRNA was subsequently measured by real-time polymerase chain reaction. D, To determine whether ceramide could restore the amplified inflammation when ceramide synthesis was blocked using pharmacological inhibitors, C2 ceramide was also added to cells that had been treated with Triacin-C. Data are mean±SEM; n=3–4. *P<0.05 vs LPS alone and vs PA or C2 ceramide alone (A and C), vs control (B), or vs Triacin-C alone (D). †P<0.05 vs PA+LPS. ‡P<0.05 vs PA.
amplification of TLR signaling, although they do not rule out contributions from other SFA metabolites.

Ceramides activate members of the protein kinase C (PKC) family. In THP-1 monocytes exposed to PA, both conventional PKC and PKC-ζ were activated (as measured by translocation to the cell membrane and phosphorylation on Ser11, respectively; Figure 3A). Similar PKC activity was observed when cells were exposed to C2 ceramide but not to C2 dihydrceramide. Importantly, the activation of conventional PKC and PKC-ζ was inhibited by Triacin-C. Interestingly, the pan-conventional/novel PKC inhibitor Calphostin-C did not inhibit amplification of the LPS response, and the potent pan-conventional/novel PKC agonist PMA did not induce any synergistic amplification of THP-1 responses to LPS (Figure 3B). In contrast, exposure of monocytes to PA in the presence of an inhibitory PKC-ζ pseudosubstrate peptide29 inhibited the ability of PA to amplify monocyte proinflammatory gene expression in response to LPS, demonstrating the requirement for PKC-ζ activity in this amplification mechanism.

Because PKC can serve as potent mediators of cell signaling events via mitogen-activated protein kinase (MAPK) pathways, we determined whether SFA activated MAPK signaling via ceramide-dependent and PKC-ζ-dependent pathways. Flow cytometry studies using activation state-specific antibodies to MAPK demonstrated that exposure of monocytes to PA induced activity of Erk1/2, JNK, and p38 (Figure 4A). Similarly, C2 ceramide also activated these same MAPK (Figure 4A, insets). We confirmed these flow cytometry results of MAPK activation by Western blot techniques (Figure 4B). Importantly, the low concentrations of LPS (1 ng/mL) used in these experiments did not induce detectable JNK or p38 activity in the absence of PA, although some activation of Erk1/2 was observed in response to LPS. In addition, Western blots showed activation of JNK1 but not JNK2 in response to PA. Further experiments demonstrated that phosphorylation of Erk1/2, JNK, and p38 could be suppressed by PKC-ζ inhibitor (Figure 4C) or by inhibitors of the metabolism of PA to ceramide (Figure VI). We also demonstrated PA-mediated activation of several MAPK-dependent transcription factors, including the AP-1 transcription factors c-Jun (Figure 4B), JunD, and c-Fos (not shown), and C/EBP-α and C/EBP-β (Figure 4C). PA-induced C/EBP activity was suppressed by the Erk1/2 inhibitor PD98059, the JNK inhibitor SP600125, or the p38 inhibitor SB202190, demonstrating this C/EBP activity is downstream of the MAPK. Importantly, the low concentration of LPS used in the present study did not induce phosphorylation of AP-1 or C/EBP transcription factors, in agreement with our findings that 1 ng/mL LPS had minimal ability to activate MAPK.

To confirm the necessity for MAPK activation in PA-induced amplification of TLR-mediated inflammation, exposure of monocytes to PD98059, SP600125, or SB202190 significantly reduced the ability of PA to amplify LPS-induced cytokine expression (Figure 4D). The combination of these MAPK inhibitors (each at a reduced concentration to avoid toxicity in combination) further reduced the ability of PA to amplify monocyte responses to LPS. Similar results were also obtained in freshly isolated primary human monocytes using SB202190 and SP600125 (Figure IIB), providing evidence that this same pathway may be relevant in vivo. Taken together, these results provide evidence that MAPK signaling is required for the SFA-amplified inflammation and suggest that ceramide derived from the metabolism of SFA leads to PKC-mediated activation of the MAPK.

Discussion

Work from several laboratories, including our own, has demonstrated that SFA induces monocyte/macrophage inflammation, which appears mediated in part by TLR4-signaling. The present study now demonstrates the novel finding that exposure of monocytes to elevated concentrations of SFA greatly amplifies the proinflammatory responses of these cells to TLR ligands though a separate TLR4-independent pathway, dependent on the uptake and metabolic processing of SFA into ceramide.

Because elucidation of this pathway relied in part on the use of 4 pharmacological inhibitors of different enzymatic steps in the conversion of PA to ceramide, and because all inhibited amplification of the inflammatory response to LPS, it is highly unlikely that these results could be attributed to nonspecific inhibitor effects. Importantly, C2 ceramide could bypass the pharmacological inhibitors of SFA metabolism and augment the inflammatory action of LPS, further demon-strating the specificity of the inhibitor effects and the importance of ceramide in this pathway. We also demon-

Figure 3. Activation of PKC-ζ is required for PA-induced amplification of LPS responses in monocytes. A, Monocytes were exposed to media (C), 100 μmol/L PA (PA), PA plus 100 μmol/L Triacin-C (PA TriC), 50 μmol/L C2 dihydrceramide (dC2), or 50 μmol/L C2 ceramide (C2). Markers of the activation of conventional PKC (translocation to the plasma membrane) and PKC-ζ (phosphorylation) were measured by Western blot. B, Monocytes were exposed to PA, LPS, or PA and LPS, in the presence or absence of PKC-ζ inhibitor (15 μmol/L; z), Calphostin-C (250 nm; CaC), or phorbol myristate acetate (100 ng/mL; PMA). IL-6 (white bars) and IL-8 (black bars) mRNA were measured by real-time polymerase chain reaction. Data are mean±SEM; n=3–4. *P<0.05 vs C, vs PA alone, or vs LPS alone. †P<0.05 vs PA+LPS. Blots are representative of 3 independent experiments.
strated activation of PKC-ζ and MAPK signaling pathways when monocytes were exposed to SFA, and we showed that inhibitors of ceramide synthesis also inhibited these pathways. Moreover, direct inhibition of PKC-ζ and MAPK also attenuated the amplified inflammation. These data provide strong support for our hypothesis that SFA-enhanced inflammation is mediated by the conversion of SFA to ceramide and subsequent ceramide-mediated activation of PKC-mediated and MAPK-mediated signaling, and it is therefore unrelated to any ability of SFA to act as TLR2 or TLR4 ligands.14,15,31

Experiments also demonstrated that PKC-ζ inhibitor simultaneously inhibited PA-induced MAPK activity, providing strong evidence that MAPK lie downstream of PKC-ζ. Although our data also showed activation of conventional PKC by PA and C2 ceramide, blockade of conventional PKC isoforms with Calphostin-C did not inhibit the ability of PA to amplify monocyte responses to LPS. Activation of conventional PKC using PMA also did not induce a synergistic amplification of the monocyte response to LPS. These data suggest conventional PKC do not contribute to the enhanced LPS response, but they do not rule out other roles for these enzymes.

As further evidence of MAPK involvement, we also confirmed that inhibitors of MAPK activity simultaneously reduced the SFA-stimulated activity of MAPK-dependent AP-1 and C/EBP transcription factors and the amplification of the LPS response. Additionally, these data may also provide insight into how MAPK activation could enhance LPS signaling. Several inflammatory cytokines, including IL-6 and IL-8, have promoter binding sites for members of the AP-1 and the C/EBP families, both of which have been reported to interact cooperatively with NF-κB to upregulate gene expression.32,33 We speculate that the observed MAPK-mediated amplification of cytokine gene expression therefore may be affected via these same transcription factors enhancing the transcriptional effectiveness of LPS/TLR4-induced NF-κB activity.

This has several important implications because NF-κB is widely recognized as a “master switch” of inflammation.8 Therefore, we speculated that SFA may enhance transcription of a wide variety of inflammatory genes in a wide variety of cell types. Exposure of monocytes to PA amplified LPS-induced expression of several genes, including IL-1β, IP-10, MCP-1, Resistin, and tumor necrosis factor-α, but not COX-2 (Figure VII), suggesting that this response is widespread among NF-κB–mediated proinflammatory genes. This also raises the possibility that SFA may prime cells to amplify...
responses not only to LPS but also to a variety of other proinflammatory stimuli that activate NF-κB. Consistent with this concept, we have also shown that exposure of monocytes to PA also amplifies their responses to the TLR2 ligand Pam3CSK4. Importantly, whereas siRNA-mediated knockdown of TLR4 does inhibit PA-amplified responses to LPS (the expected result because cells lacking TLR4 cannot respond to LPS), there is no inhibition of the ability of PA to amplify monocyte responses to the TLR2 ligand Pam3CSK4. This result clearly shows that PA-mediated amplified inflammation is not dependent on a specific TLR but must operate through a separate and more general mechanism. We illustrate in schematic form (Figure VIII) the pathway that we believe our data suggest underlies this amplification.

The synergy between LPS and FFA was specific for SFA (PA, lauric acid, and stearic acid) and was not found when THP-1 monocytes were exposed to unsaturated FFA. In agreement with earlier reports,15,31 linoleic acid mildly suppressed the responses of monocytes to TLR ligands. Oleic acid neither amplified nor inhibited the responses of monocytes to LPS. This may be because of the preferential conversion of SFA to ceramide34 or to the ability of unsaturated FFA to inhibit the underlying LPS-induced inflammation.14,30 However, the amplification effect was maintained for THP-1 cells and primary human monocytes when SFA were mixed with unsaturated FFA in physiological concentrations. These data are consistent with previous reports that SFA, but not unsaturated FFA, were proinflammatory.14–16,22,31 Although a previous report indicated that lauric acid was not directly capable of activating TLR-mediated signaling in mouse RAW296.7 macrophages,30 this finding actually supports our interpretation that SFA-enhanced inflammation occurs via a novel mechanism independent of direct SFA/TLR interactions. Importantly, the present results were obtained in human cells while using fatty acids and LPS at plasma levels commonly found in individuals with obesity or type 2 diabetes, or after consumption of high-fat meals,35–39 thereby providing evidence that the ability of SFA to amplify TLR signaling may have important physiological implications.

Consistent with this, our data also show that PA enhances the LPS response of macrophages and monocytes. Macrophage accumulation in tissue and production of proinflammatory cytokines are believed critical in development of insulin resistance, diabetes, and cardiovascular disease. We have previously demonstrated that cytokines released from macrophages induced adipocyte inflammation and reduced insulin action and lipolysis.6 The resulting increase in FFA released from adipocytes can further enhance the macrophage-initiated proinflammatory cascade.

Several methodological considerations deserve mention. Although the present study is consistent with in vivo studies linking excess nutrition and elevated plasma SFA concentrations to inflammatory consequences,16,36,40 our results were largely obtained using human cell lines. For this reason, the existence of this synergistic effect, its dependence on SFA uptake, and its dependence on MAPK activity were also confirmed ex vivo in primary human monocytes. The decision to conduct these experiments with human cells and cell lines had advantages and disadvantages. Using human cells (monocytes and macrophages) provided considerably more weight to the potential relevance of this phenomenon to human disease. More importantly, whereas it might prove fruitful to examine specific signaling steps in this amplification pathway using mouse models in the future, initial experiments exposing mouse RAW296.7 macrophages to PA did not result in an amplified response to LPS (data not shown). These preliminary data suggested differences in responses between mouse and human cells, possibly reflecting well-known species differences in the sensitivity and specificity of human and murine TLR to certain ligands.41 However, the necessity of using human monocytes limited the availability of genetically modified (eg, knock-out) models and generally complicated the use of molecular methods. For example, whereas we successfully used siRNA to knock-down TLR4, our attempts to use siRNA against serine palmitoyltransferase consistently led to considerable cell death and a minimally effective knockdown in the remaining cells. Consistent with our experience, Tamehiro et al42 still needed to use Myricin to achieve substantial inhibition of ceramide synthesis even in THP-1 cells selected (using puromycin) for successful transfection and expression of lentiviral shRNA against this enzyme. To overcome these difficulties, we studied this pathway using multiple, functionally unrelated inhibitors.

In conclusion, this study presents the novel finding that SFA can greatly amplify the inflammatory responses of monocyte/macrophages to LPS via TLR4-independent mechanisms. These data suggest that uptake of SFA leads to enhanced ceramide generation, which in turn activates PKC-ζ and MAPK. Concentrations of SFA and LPS that are commonly present in conditions resulting from excess nutrition (eg, obesity, insulin resistance, type 2 diabetes, etc) and high-fat diets can lead to inflammation that is several-fold greater than the direct proinflammatory effects of each of these factors alone. The elevation of SFA and LPS in these settings could create a “perfect storm” of factors leading to greatly increased monocyte/macrophage innate immune responses. Because monocytes/macrophages are critical cells mediating tissue and systemic inflammation, a broad and “synergistic” amplification of their inflammatory activity by SFA may be an important contributor to the genesis or progression of insulin resistance and type 2 diabetes, as well as the cardiovascular sequelae of these conditions.

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Disclosure

Y.S. is employed by Kronos Science Laboratory. This work does not represent the views of the Department of Veterans Affairs or the United States Government.
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SUPPLEMENT

SUPPLEMENTARY METHODS

Materials and Reagents

THP-1 cells were purchased from ATCC (Gaithersburg, MD). Cell culture media and supplements were from Invitrogen (Gaithersburg, MD). C2 ceramide and dihydroceramide were from Biomol (Plymouth Meeting, MA). Ceramide standards for mass spectrometry were from Avanti Polar Lipids (Alabaster, AL). PKC-ζ pseudosubstrate inhibitor was from Tocris Bioscience (Ellisville, MO). PD98059, and SB202190 were from VWR (South Plainfield, NJ). ELISAs were from R&D Systems (Minneapolis, MO). DNA-binding ELISAs were from Active Motif (Carlsbad, CA). Flow cytometry reagents were from Beckman-Coulter (Fullerton, CA). Real-time PCR reagents were from Bio-Rad (Hercules, CA). Antibodies to phosphorylated and total proteins were from Cell Signaling Technology (Danvers, MA). Antibody to TLR4 (clone HTA125) was from eBioscience (San Diego, CA). Specific monoclonal antibody to activated NF-κB (clone 12H11) was from Millipore (Billerica, MA). Specific monoclonal antibody to CCR2 (clone TG5/CCR2) was from Biolegend (San Diego, CA). All other reagents not otherwise specified were from Sigma-Aldrich (St. Louis, MO).

Cell culture and experimental design

THP-1 monocytes (passage 5-15) were routinely cultured in growth media (RPMI-1640 media supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium
pyruvate, 100 mM HEPES, and 20 μM β-mercaptoethanol) under standard cell culture conditions (37 °C, humidified, 95% air / 5% CO₂), and reseeded every 2-3 days to maintain a density of 250,000 to 1,000,000 cells/mL. All reagents were frequently tested for the presence of LPS using a high-sensitivity Limulus amoebocyte assay kit (Lonza, Walkersville, MD).

For experiments on inflammation responses, cells were reseeded in growth media with or without FFA, LPS, specific pharmacologic inhibitors, and/or combinations of these reagents. For all reagents not part of the routine culture of THP-1 cells, we performed Trypan Blue exclusion and lactose dehydrogenase toxicity assays (Sigma) in the presence of PA, and reagent concentrations used in this manuscript were not associated with observable cell toxicity (data not shown). Cells were typically incubated with FFA and/or inhibitors for 16 hours, washed in PBS, and resuspended in fresh growth media with or without 1 ng/mL E. coli LPS for 30 minutes (for phosphoprotein assays), 4 hours (for real-time PCR), or 48 hours (for conditioned media ELISA). In other experiments, the TLR2 ligand Pam3CSK4 (5 ng/mL; Invivogen, San Diego, CA) was substituted for LPS.

To produce THP-1 macrophages, aliquots of 1,000,000 monocytes were seeded in 2 mL of growth media supplemented with 100 ng/mL phorbol ester for 24 hours, then subsequently washed to remove non-adherent cells. Adherent cells were allowed to mature into macrophages for 7 days prior to experiments, and were fed with 2 mL growth media every 2 days.
Human monocytes were prepared from blood donated as part of a general laboratory specimen collection protocol previously approved by the IRB of the Phoenix VA Health Care System. Monocytes were isolated using dual Histopaque-1077 and Percoll density gradient centrifugation, as previously described. Human monocytes were maintained in modified growth media containing 10% human type AB serum (Sigma) in place of fetal bovine serum.

**Transfection**

THP-1 monocytes were transfected using the Amaxa Nucleofector II and Nucleofector Kit V (Lonza, Walkersville, MD), and following manufacturer’s protocols for THP-1 monocytes and optimized for our genes of interest. Briefly, THP-1 cells were pelleted by centrifugation at 1000 RPM for 10 minutes and all media was aspirated from the pellet. The cells were subsequently resuspended by gentle flicking to a density of 10,000,000 cells/mL in Nucleofector V transfection solution, and aliquots were mixed with siRNA to TLR4 (5 µg/mL; Dharmacon, Lafayette, CO), or SPTLC1 (5-10 µg/mL; constructs from both Dharmacon, and SA Biosciences, Frederick, MD), or with non-targeting control siRNA (Dharmacon). Aliquots of 1,000,000 cells were eletroporated using Nucleofector program V-001, and were immediately transferred to 500 mL cell growth media. All aliquots were transfected within 15 minutes of cell resuspension, and allowed to recover for 20 minutes. Cells were then transferred to 2 mL of growth media and incubated for 3 days to deplete internal stores of targeted protein. Cells transfected with the same siRNA were pooled to ensure uniformity of subsequent aliquots before being used in
Fatty acid preparation

Because we had previously found many common laboratory reagents were contaminated with LPS, FFA were mixed to a final concentration of 40 mM in a solvent of 0.1 N NaOH / 70% ethanol, and heated to 70 °C until dissolved. This alkaline buffer hydrolyzes and detoxifies > 10 ng/mL LPS \(^2\) and > 2.5 ng/mL of the lipopeptide analogues FSL and Pam3CSK4 (unpublished data). In addition, because we had previously detected ubiquitous endotoxin contamination of even low-endotoxin preparations of bovine serum albumin, we added our FFA directly to the cell culture media, where they could complex with the bovine serum albumin already present in the 10% fetal bovine serum. Except as otherwise noted, individual FFA were added at a final concentration of 100 \(\mu\)M. The mixture of FFA used in some confirmatory experiments was 30% palmitic acid, 10% stearic acid, 32% oleic acid, 20% linoleic acid, 1% eicosapentaenoic acid, and 1% docosahexaenoic acid (molar %), and was used at 200 \(\mu\)M final concentration. The final FFA:albumin ratio used in experiments was approximately 2.3:1.

Real-time PCR

RNA was extracted from cells using the Aurum mini column method (Bio-Rad Laboratories, Hercules, CA) and following manufacturer’s instructions. Reverse transcriptase PCR was performed using Bio-Rad iScript reagent. Real-time, semi-quantitative PCR was performed on a Bio-Rad iCycler iQ instrument, using SYBR Green
chemistry (Bio-Rad) and primers as previously described ³.

**Ceramide measurements**

Aliquots of 1x10⁶ monocytes were collected by centrifugation and washed twice in PBS to remove extracellular lipids. Cell pellets were spiked with an internal standard of 25 ng deuterated C16-d31 ceramide. Total polar lipids were extracted from each cell pellet using chloroform:methanol (2:1) and supernatants were dried under nitrogen. Dried lipids were resuspended in water/methanol mobile phase and analyzed using LC/MS-MS on an API2000 triple stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA) with the positive electrospray ionization method using the multiple reaction monitoring mode (ion pairs of 538.4/264.4, 566.4/164.4, and 569.3/265.4 were used to monitor C16, C18 and C16-d31, respectively). Concentrations of these ceramides were quantified by comparison of the ratio of the mass chromatogram peak areas of each ceramide vs. C16-d31 with a 7-point external standard calibration curve for each ceramide, using Analyst 1.41 software.

**ELISA**

DNA binding activity of AP-1 and C/EBP-α and –β was measured using DNA-binding ELISA kits (Active Motif, Carlsbad, CA) following manufacturer’s instructions, using a VersaMax plate reader (Molecular Dynamics, Sunnyvale, CA). IL-6 and IL-8 proteins were similarly measured by ELISA (R&D Systems; Minneapolis, MN).

**Western blots**
Activity of mitogen-activated protein kinases (MAPK) was measured using Western blots, following standard laboratory methods using NuPAGE reagents (Invitrogen). Phosphoprotein bands were visualized using primary antibodies against phosphorylated Jun N-terminal kinase-1 and -2 (JNK1/2), p38 MAPK, and extracellular-regulated kinase-1 and -2 (Erk1/2), as well as phosphorylated c-Jun, and β-actin (Sigma).

**Flow Cytometry**

To measure cell surface expression of TLR4 and CD14, THP-1 monocytes were fixed in 4% paraformaldehyde in PBS for 30 minutes. Cells were subsequently washed in PBS, then blocked in PBS containing 2% fetal bovine serum for 10 minutes. CCL2, TLR4 and CD14 were labeled using specific monoclonal antibodies (clones TG5/CCL2, HTA125, and 61D3, respectively), followed by biotinylated secondary antisera (eBioscience). Cells were stained using allophycocyanin-labeled streptavidin (SouthernBiotech, Birmingham, AL). TLR4 binding activity was measured by exposing unfixed monocytes to 1, 10, and 100 ng/mL fluorescein isothiocyanate-labeled LPS (Sigma) for 30 minutes; preliminary experiments showed no concentration-related difference in LPS binding, and data are presented using 10 ng/mL. MAPK activity was also assayed using fluorescently-labeled, activation-state-specific antibodies to p38, JNK, or Erk (Beckman-Coulter, Fullerton, CA), following manufacturer’s instructions. NF-κB activity was measured using a specific monoclonal antibody that recognizes the exposed IκB-binding site on the activated NF-κB p65 subunit (clone 12H11). Staining was as for TLR4, except that cells were fixed in 3.7% formaldehyde for 10 minutes, then permeabilized in 90% methanol for 30 minutes prior to the blocking step. Fluorescent labeling of TLR4 and CD14
surface expression, fluorescein isothiocyanate-labeled LPS binding, as well as MAPK and NF-κB activity was measured on an FC500 flow cytometer (Beckman-Coulter).

**Statistics**

Except as otherwise noted, experiments were repeated at least in triplicate. Results consisting of raw numerical data (e.g., DNA-binding ELISA) were analyzed by ANOVA followed by post-hoc Tukey tests. Results consisting of samples normalized to same-experiment controls (e.g., real-time PCR) were analyzed using Z-statistics with Bonferroni’s correction. Data were presented as mean ± SEM; significance was taken as p ≤ 0.05.

**SUPPLEMENTARY RESULTS**

Our central results, namely that the saturated fatty acid PA amplifies TLR signaling in a human monocyte cell line, were confirmed and expanded upon with other saturated fatty acids and different cells of monocytic lineage. First, lauric acid and stearic acid induced effects similar to those caused by PA (Fig. 1). In contrast, exposure of cells to the unsaturated fatty acids linoleic acid or oleic acid either suppressed or failed to enhance (respectively) IL-6 and -8 mRNA responses to LPS (Suppl. Fig. I). It is important to note that amplification of the response to LPS was also seen when the monocytes were incubated with an elevated concentration of a mixture of FFA approximating the distribution of saturated and unsaturated FFA in human plasma (Suppl. Fig. IIA).
Confirmatory experiments performed with primary human monocytes pre-treated with this FFA mixture also showed ~4.5-fold amplification of LPS-induced IL-6 and a nearly 3-fold increase in IL-8 expression (Suppl. Fig. IIB) compared to LPS alone. Importantly, Triacsin-C inhibited the ability of physiological mixtures of FFA to amplify LPS-induced inflammation in primary human monocytes ex vivo (Suppl. Fig. II B), suggesting that the same ceramide-dependent mechanism demonstrated in THP-1 cells might also be relevant in vivo. Similarly, exposure of THP-1 macrophages (differentiated from THP-1 monocytes using 100 μM phorbol ester) to PA induced a reproducible increase in LPS-stimulated IL-6 and IL-8 mRNA levels, although this required slightly longer incubation (24 hours) with PA (Suppl. Fig. IIC).

Importantly, siRNA to TLR4 inhibited the response of the monocytes to LPS, but did not alter either the response of the cells to Pam3CSK4 or the amplification of that response by PA (Suppl. Fig. III). These supplementary data demonstrate that PA amplification of TLR signaling does not depend on TLR4 specifically, and points out the distinct possibility that SFA may amplify inflammatory signal pathways for multiple TLRs.

To elucidate the underlying mechanism(s) by which SFA amplifies monocyte TLR-dependant inflammation, we first examined several obvious possibilities. Both Western blot (data not shown) and flow cytometry studies showed no increase in total or cell surface TLR4 expression (Suppl. Fig. IV A). Similar results were observed for CD14 (data not shown). Following PA treatment, LPS binding activity was actually slightly decreased (Suppl. Fig. IV B). Furthermore, although we previously reported that exposure of monocytes to PA alone induced NF-κB activity 1, 2, PA-induced NF-κB
activity was not additive with that induced by LPS (Suppl. Fig. IV C). These data indicated that (even if SFA are themselves a weak TLR4 ligand, as suggested by some 4, 5, but not all 6 investigators) the ability of SFA to amplify LPS-induced TLR4-mediated inflammation is not due to upregulation of TLR4 expression or activity. Monocytes exposed to PA showed no morphological changes consistent with macrophage differentiation, and in fact demonstrated increased gene expression (with no change in protein) of CCR2, a receptor that characteristically decreases in expression during differentiation into macrophages (Suppl. Fig. V) These data suggest that PA does not increase inflammation by inducing differentiation of the monocytes to macrophages, a conclusion further strengthened by the observation that PA markedly amplifies LPS-induced inflammation in cells already differentiated to macrophages (Suppl. Fig. IIC).

SUPPLEMENTARY REFERENCES

SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure I. Exposure of monocytes to unsaturated FFA does not amplify subsequent responses to LPS.  
A. THP-1 monocytes were exposed to media alone (C), or to 100 μM of linoleic acid (LA) or oleic acid (OA) for 16 hours, followed by exposure to 1 ng/mL LPS (LPS) for 4 hours as described in Methods. IL-6 (white bars) and IL-8 (black bars) mRNA was measured by real-time PCR. Data are mean ± SEM; n = 3.

Supplementary Figure II. Exposure of cultured or primary human monocytes, or cultured human macrophages, to physiological mixtures of SFA in combination with unsaturated FFA also show amplified responses to LPS.  
A. THP-1 monocytes were exposed to media for 16 hours (C), media followed by 1 ng/mL LPS for 4 hours (LPS), 200 μM non-esterified fatty acids (NEFA; fatty acid composition as described in Methods) for 16 hours, or NEFA followed by LPS (NEFA LPS). Expression of cytokine (IL-6 and IL-8) mRNA was measured by real-time PCR.  
B. Freshly-isolated primary human monocytes were also exposed to NEFA and/or LPS similar to the THP-1 cells, above. Additionally, primary human monocytes were exposed NEFA and LPS in the presence of the acyl-CoA transferase inhibitor Triacsin-C (TriC), the p38 MAPK inhibitor SB202190 (SB), and the JNK inhibitor SP600125 (SP).  
C. Similar experiments were also performed exposing THP-1 macrophages (differentiated with 100 ng/mL phorbol ester) to PA for 24 hours followed by 1 ng/mL LPS for 4 hours. Data are mean ± SEM; n = 3 - 6; * p < 0.05 both vs. LPS alone and vs. PA or NEFA alone; † p < 0.05 vs. NEFA LPS.
Supplementary Figure III. PA amplifies TLR2 responses, and siRNA-mediated knockdown of TLR4 does not affect the amplification response. THP-1 monocytes were sham-transfected with a non-targeting siRNA (black bars) or with siRNA against TLR4 (grey bars; knockdown efficiency 60% by qPCR). Cells were subsequently maintained under control conditions (C), or exposed to PA, the TLR4 ligand LPS (LPS), or the TLR2 ligand Pam3CSK4 (Pam3), alone or in combination. Pre-treatment with PA amplified monocyte responses to Pam3CSK4 (PA Pam3) as well as LPS. Knockdown of TLR4 inhibited monocyte responses to LPS, however, monocyte responses to Pam3CSK4, and amplification of Pam3CSK4 response by PA, were unaffected. Data are mean ± SEM; n = 3; * p < 0.05 both vs. LPS or Pam3 alone.

Supplementary Figure IV. Exposure of monocytes to SFA did not amplify TLR4 expression, LPS binding capacity, or LPS-stimulated NF-κB activity. THP-1 monocytes were exposed to media (C-black histograms), or media containing 100 μM palmitic acid (PA-white histograms) for 16 hours. A. TLR4 surface expression was measured by flow cytometry using a monoclonal antibody to TLR4 and a biotin/allophycocyanin-streptavidin labeling system. Similar results were also found for CD14 surface expression (not shown). B. Aliquots of these cells were also stained with fluorescein isothiocyanate-labeled LPS for 30 minutes. LPS binding was measured using flow cytometry. C. Cells were exposed to media for 16 hours (C; and the control image replicated for comparison in other frames), media followed by 1 ng/mL LPS for 30 minutes (LPS), 100 μM PA for 16 hours (PA), or PA followed by LPS (PA LPS). NF-
κB activity was measured by flow cytometry using a monoclonal antibody specific to activated NF-κB. Histograms and data are representative from n ≥ 3 independent experiments counting 10,000 cells each; all histograms in C. are from the same representative experiment.

Supplementary Figure V. Exposure of monocytes to SFA does not induce differentiation of monocytes to macrophages. To confirm that the amplified expression of proinflammatory genes wasn’t due to SFA-mediated transformation of the monocytes to macrophages, THP-1 monocytes were exposed to media (C) or palmitic acid (PA) for 16 hours. Expression of the monocyte marker CCR2 was measured by flow cytometry (white bars) and real-time PCR (black bars). Data are mean ± SEM; n = 3.

Supplementary Figure VI. Pharmacologic antagonists of the metabolism of PA to ceramide inhibit PA-induced MAPK phosphorylation. Monocytes were maintained in media (C) or exposed to 100 μM palmitic acid (PA) for 16 hours in the presence or absence of the serine palmitoyltransferase inhibitor Myriocin (Myr), the ceramide synthase inhibitor Fumonisin B1 (FB1), the ketosphinganine synthetase inhibitor L-cycloserine (LCS), or the acyl-CoA synthetase inhibitor Triacsin-C (TriC). Phosphorylation of the mitogen-activated protein kinases p38, JNK, and Erk1/2 was measured by flow cytometry. Data are mean ± SEM; n = 3; * p < 0.05 vs. PA.

Supplementary Figure VII. LPS responses of multiple proinflammatory cytokine genes are upregulated by exposure of monocytes to PA. THP-1 monocytes were
exposed to media (C-black histograms), or media containing 100 μM palmitic acid (PA-white histograms) for 16 hours, then were subsequently exposed to LPS for 4 hours. Expression of expression of IL-1β, IP-10, Resistin, and mRNA was measured by real-time PCR. In addition, MCP-1 and TNF-α (not shown) demonstrated trends towards amplification but did not reach significance vs. PA and/or LPS. Data are mean ± SEM; n = 3; * p < 0.05 both vs. LPS alone and vs. PA alone; † p < 0.05 vs. the sum of PA alone + LPS alone.

Supplementary Figure VIII. A schematic of a proposed amplification mechanism.

TLR4 binds LPS and activates NF-κB-mediated gene expression. However, in the presence of physiologically relevant doses of SFA, excess SFA are converted to ceramide, which subsequently activates PKC-ζ. PKC-ζ then activates the MAPK signaling pathway, leading to activation of MAPK-dependent transcription factors such as c-Jun/AP1 and the C/EBP family. These transcription factors then enhance the transcriptional effectiveness of NF-κB, leading to greatly enhanced expression of several proinflammatory genes. Solid arrows indicate synthesis steps; dashed arrows indicate activation steps; diamond boxes represent kinases; arrow boxes represent transcription factors.
Supplementary Figure I
Supplementary Figure II

A

B

C

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Supplementary Figure III

A

Gene Expression
(fold induction vs. control)

IL-6

sham

siTLR4

B

Gene Expression
(fold induction vs. control)

IL-8

sham

siTLR4

C

PA

LPS

PALPS

Pam3

PAPam3

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Supplementary Figure IV

A

Cell Number (events)

TLR4 Surface Expression

log (RLU)

B

Cell Number (events)

FITC-LPS Binding

log (RLU)

C

Cell Number (events)

Control

PA

LPS

PA LPS

Cell Number (events)

NF-κB Activity

log (RLU)
Supplementary Figure V

![Supplementary Figure V](image)

- **X-axis:** C, PA
- **Y-axis:** CCR2 Expression (fold induction vs. control)
- **Legend:**
  - Protein
  - mRNA

This figure shows the expression levels of CCR2 in response to two different conditions: C and PA. The mRNA expression is significantly higher in the PA condition compared to the control (C) condition.
Supplementary Figure VI

The figure shows a bar graph representing MAPK activity (RLU) for different conditions and treatments.

- **p38**
- **JNK**
- **Erk**

The conditions and treatments include:
- C
- PA
- PA Myr
- PA FB1
- PA LCS
- PA TiC

The graph indicates changes in MAPK activity across these conditions with significance marked by asterisks (*) for certain treatments.
Supplementary Figure VII

![Graph showing cytokine gene expression](image)

- IL-1β
- IP-10
- MCP-1
- Resistin
- TNF-a

Cytokine Gene Expression (fold induction vs. control)

- C
- PA
- LPS
- PA LPS

* Indicates significant difference
Supplementary Figure VIII

[Diagram showing the relationship between SFA, LPS, TLR4, and various signaling pathways such as PKC-ζ, Erk1/2, p38, JNK1, AP-1, C/EBP, and NF-κB leading to increased inflammatory gene expression.]