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Inflammasome-Mediated Secretion of IL-1β in Human Monocytes through TLR2 Activation; Modulation by Dietary Fatty Acids

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Many studies have shown that TLR4- and TLR2-deficient mice are protected from high-fat diet–induced inflammation and insulin resistance, suggesting that saturated fatty acids derived from the high-fat diet activate TLR-mediated proinflammatory signaling pathways and induce insulin resistance. However, evidence that palmitic acid, the major dietary saturated fatty acid, can directly activate TLR has not been demonstrated. In this article, we present multiple lines of evidence showing that palmitic acid directly activates TLR2, a major TLR expressed on human monocytes, by inducing heterodimerization with TLR1 in an NADPH oxidase–dependent manner. Dimerization of TLR2 with TLR1 was inhibited by the n-3 fatty acid docosahexaenoic acid. Activation of TLR2 by palmitic acid leads to expression of pro–IL-1β that is cleaved by caspase-1, which is constitutively present in monocytes, to release mature IL-1β. Our results reveal mechanistic insight about how palmitic acid activates TLR2, upregulates NALP3 expression, and induces inflammasome-mediated IL-1β production in human monocytes, which can trigger enhanced inflammation in peripheral tissues, and suggest that these processes are dynamically modulated by the types of dietary fat we consume.
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Chronic inflammation is one of the key causative conditions for the development and progression of many chronic diseases. Low-grade chronic inflammation characterized by elevated circulating concentrations of proinflammatory cytokines, acute-phase proteins, and adhesion molecules is known to be associated with obesity and insulin resistance. What causes and mediates low-grade chronic inflammation and how it can be suppressed through dietary means that can provide preventive efficacy for inflammatory chronic disease are challenging questions.

Blood monocytes are sentinel immune effector cells that detect and respond to invading pathogens and dietary components absorbed from the gut. Activated blood monocytes can extravasate into peripheral tissues and become resident macrophages or dendritic cells (DCs) that can trigger inflammatory signals in response to exogenous and endogenous stimuli (1, 2). Recent animal studies have shown that a high-fat diet increases infiltration of macrophages and DCs, which originate from circulating blood monocytes, in adipose tissue where they play a central role in the development of chronic inflammation and insulin resistance (3). Therefore, activation of proinflammatory pathways in circulating blood monocytes is the gateway toward enhanced inflammation in various tissues. IL-1β is a major cytokine involved in the activation of blood monocyte and proinflammatory signaling pathways in peripheral tissues. Unlike other proinflammatory cytokines, IL-1β production is tightly regulated by a unique two-signal mechanism. The primary signals induce the expression of pro–IL-1β mediated, in part, through the activation of TLRs. The secondary signals activate the NALP3 inflammasome, an intracellular signaling complex composed of NALP3, procaspase-1, and the adaptor protein ASC. Formation of the inflammasome complex results in proteolytic cleavage of procaspase-1 to yield active caspase-1, which, in turn, cleaves pro–IL-1β producing mature and active IL-1β. Mature IL-1β released extracellularly binds its cognate receptor in a paracrine manner, resulting in amplification of proinflammatory responses (4–7). However, recent findings suggest that the two-signal model for NALP3 inflammasome-mediated IL-1β secretion may not be applicable to all IL-1β–secreting cells including blood monocytes and DCs. Tissue macrophages require both primary and secondary signals for NALP3 inflammasome-mediated IL-1β secretion. In contrast, blood monocytes have constitutively active NALP3 inflammasome; therefore, TLR stimulation alone is sufficient to induce inflammasome-mediated IL-1β production in monocytes (8–10). Similarly, both bone marrow–derived and splenic DCs secrete substantial amounts of inflammasome-mediated IL-1β upon TLR activation in the absence of secondary signals (3, 11). Therefore, regulation of NALP3 inflammasome-mediated IL-1β production in mononuclear phagocytes appears to be cell-type specific.

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Abbreviations used in this article: BMDC, bone marrow–derived dendritic cell; BMDM, bone marrow–derived macrophage; DC, dendritic cell; DHA, docosahexaenoic acid; FRET, fluorescence resonance energy transfer; LPL, lipoprotein lipase; Nod, nucleotide-binding oligomerization domain–containing protein; NOX2, NADPH oxidase 2; Pam_3CSK_4, Pam3CysSerLys4; PRR, pattern recognition receptor; PUFA, polyunsaturated fatty acid; ROS, reactive oxygen species; SFA, saturated fatty acid; siRNA, small interfering RNA; TGRL, triglyceride-rich lipoprotein; TR-FRET, time resolved FRET.

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Pattern recognition receptors (PRRs) induce innate immune responses by recognizing invariant pathogen-associated molecular patterns leading to activation of downstream signaling pathways and the expression of a diverse array of proinflammatory gene products that are required for host defense against invading pathogens. In addition, PRRs are activated by endogenous molecules derived from tissue injury and elicit sterile inflammation to initiate wound-healing processes (12, 13). Activation of PRRs can also be modulated by dietary components and their metabolites bridging immune responses to metabolic homeostasis (3, 14–19).

Our previous studies first revealed that a medium-chain saturated fatty acid (SFA), lauric acid, can activate TLR4, TLR2, and nucleotide-binding oligomerization domain–containing protein (Nod) 2–mediated signaling pathways, whereas the n-3 fatty acid, docosahexaenoic acid (DHA), inhibits lauric acid–induced activation of TLRs or Nod-mediated signaling pathways (15, 20–26). Many studies with animal models also showed that TLR4 or TLR2 deletion or mutant mice were protected from high-fat diet–induced inflammation and insulin resistance (3, 14, 16, 27–31), suggesting that high saturated fat diet activates TLR-mediated proinflammatory signaling pathways and induces insulin resistance. However, evidence that SFAs derived from high-fat diet can directly activate TLRs in humans has not been demonstrated. In this article, we present multiple lines of evidence that palmitic acid, the major dietary SFA, directly activates TLR2, a predominant TLR expressed on blood monocytes, by inducing heterodimerization with TLR1. The dimerization of TLR2 with TLR1 as assessed by time resolved-fluorescence resonance energy transfer (TR-FRET) was inhibited by DHA. Activation of TLR2 leads to expression of pro–IL-1β that is cleaved by caspase-1, which is activated by constitutively active inflammasome, to release mature IL-1β in blood monocytes. Our results reveal mechanistic insight about how the SFA, palmitic acid, induces inflammasome-mediated IL-1β production and suggest that inflammasome-mediated IL-1β production in human blood monocytes, which can trigger enhanced inflammation in peripheral tissues, is dynamically modulated by the types of dietary fat we consume.

Materials and Methods

Reagents

LPS (catalog no. 421) was purchased from List Biological Laboratories (Campbell, CA). Pam3CysSerLys4 (Pam_3CSK_4; catalog no. tlrl-pms) and TAK-242 (catalog no. tlrl-cli95) were purchased from Invivogen (San Diego, CA). BSA (catalog no. 30-AB79, Lot no. A10072001) was purchased from Fitzgerald Industries International (Acton, MA). Palmitic acid (catalog no. P5585), sodium palmitate (catalog no. P9767), sodium salt DHA (catalog no. D8768), lipoprotein lipase (LPL) (catalog no. L2254), endotoxin-free water, polymyxin B (catalog no. P4932), and Ab for β-actin were purchased from Sigma (Saint Louis, MO). Apocynin (catalog no. 178385) and Ac-YVAD-AOM (catalog no. 400015) were purchased from EMD Chemicals (Darmstadt, Germany).

Cell culture

THP-1 cells (human monocytic cell line, ATCC TIB-202) were cultured in RPMI 1640 medium (ATCC catalog no. 30-2001) containing 10% (v/v) FBS (Premium Select, catalog no. S11550, Lot no. K0109 and K11050; Atlanta Biologicals, Lawrenceville, GA), 0.05 mM 2-ME, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were maintained at 37°C in a 5% CO_2/air environment. Cells were seeded at 1×10^6/ml and incubated in serum-poor medium (0.25% and 1.0% FBS) before treatment.

Fatty acid preparations

A stock solution of 100 mM sodium palmitate (C16:0) was prepared in 70% ethanol in a glass vial and heated to 60°C. Sodium palmitate was reheated to 60°C and vortexed before use to give appropriate final concentration indicated in the figures. A stock solution of 10 mM DHA sodium salt was prepared in endotoxin-free water, flushed with nitrogen, then sealed in air-tight tube and stored at −20°C until use. Palmitic acid solubilized with BSA (C16:0-BSA) was carried out as previously described (32). In brief, palmitic acid was dissolved in 100% ethanol (250 or 500 mM), then mixed with BSA in a 10:1 molar ratio in 0.25% FBS/RPMI 1640 medium. The mixture was sonicated in a water bath for 15 min, then rocked at 55°C for 15 min. BSA-solubilized palmitic acid was filtered through a 0.22-μm filter before use.

We used sodium palmitate instead of BSA-solubilized palmitic acid in our studies because we initially found that many commercially available BSA preparations contain contaminants with agonist activity for certain PRRs (32). We also found that sodium palmitate is able to stimulate PRR-mediated signaling pathways in most suspension cells such as THP-1 monocytes and primary human blood monocytes. However, palmitic acid needs to be solubilized with albumin (BSA) to stimulate PRR-mediated signaling pathways in adherent cells. After recently discovering a BSA preparation (catalog no. 30-AB79, Lot no. A10072001; Fitzgerald Industries International) that did not significantly induce PRR target gene products, we found that BSA-solubilized palmitic acid showed similar results as sodium palmitate with regard to IL-1β production in THP-1 cells (Supplemental Fig. 1).

Plasmids

Expression vectors pDisplay, pDisplay-TLR1, pDisplay-TLR2, pDisplay-TLR4, and pDisplay-TLR6 were obtained from Adeline Hajjar (University of Washington, Seattle, WA). MD2 was provided by Kensuke Miyake (Tokyo University, Japan). (2x)-NF-κB–luciferase reporter construct was provided by Frank Mercurio (Signal Pharmaceuticals, San Diego, CA). pRSV–β-galactosidase plasmid was from Jongdae Lee (University of California, San Diego, CA). The plasmid DNA from these expression vectors was prepared in large scale for transfection using the EndoFree Plasmid Maxi Kit (Qiagen, Valencia, CA).

Transfections and luciferase assays

Transient transfections were carried out using SuperFect transfection reagent (Qiagen) according to the manufacturer's recommendations. HEK293T cells were seeded at 2×10^5 per well in 24-well plates and cotransfected the following day with 10 ng each of pDisplay-TLR4 and MD2, or pDisplay-TLR2 and pDisplay-TLR1, or pDisplay-TLR2 and pDisplay-TLR6, in addition to 50 ng (2x)-NF-κB–luciferase reporter and 10 ng pRSV–β-galactosidase expression vectors. Twenty nanograms of pDisplay empty vector was used in addition to the earlier amounts of (2x)-NF-κB and pRSV–β-galactosidase for transfection as controls. Twenty-four hours after transfection, the cells were serum-starved in 0.25% FBS/DMEM medium for 6 h followed by 12-h treatment with BSA-solubilized palmitic acid in the same low serum medium. The cells were lysed. Luciferase and β-galactosidase enzyme activities were determined from the lysate supernatants using the luciferase and β-galactosidase enzyme assay system (Promega, Madison, WI) according to the manufacturer's instructions. Luciferase activity was normalized by β-galactosidase activity to correct differences in transfection efficiency among samples. Each experiment was repeated at least three times.

Immunoblot and ELISA assays

Immunoblotting was performed as previously described (17). In brief, THP-1 cells were lysed by sonication in cell lysis buffer (Cell Signaling Technology, Danvers, MA) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1 μg/ml leupeptin plus 1 mM PMSF. Lysate supernatants were collected by centrifugation and subjected to 10% or 10–20% SDS-PAGE followed by protein transfer to polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA). The membrane was blocked in TBST buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, and 0.05% [v/v] Tween 20) containing 5% nonfat milk or BSA. The membrane was probed with primary Ab for 1 h at room temperature or overnight at 4°C followed by incubation with HRP-conjugated secondary Ab (Amersham Biosciences, Piscataway, NJ) for 1 h at room temperature. Proteins were detected by ECL Western blot detection reagents (Amersham Biosciences) followed by exposure to autoradiography film (BioExpress, Kaysville, UT). Anti–IL-1β (3ZD) Ab was obtained from the National Cancer Institute Preclinical Repository; anti–caspase-1 p10 (C-20) and anti–IκBα (C-21) Abs were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-NALP3 (catalog no. ALX-804-881) Ab was purchased from Enzo Life Science (Plymouth Meeting, PA). Anti-JNK (catalog no. 9252), anti–phospho-JNK (catalog no. 9251), anti–phospho-IκBα (catalog no. 2859), and anti-MyD88 (catalog no. 4283) Abs were purchased from Cell Signaling Technology. Anti–Flotillin-1 (catalog no. 610820) and anti-$p47^{phox}$ (catalog no. 610355) Abs were purchased from

BD Biosciences (San Jose, CA). Anti-Transferrin Receptor (catalog no. 13-6890) Ab was purchased from Life Technologies Corporation (Grand Island, NY). Cell-culture supernatants were analyzed for IL-1β using BD OptEIA Human IL-1β ELISA using a Synergy 2 plate reader (BioTek, Winooski, VT) according to the manufacturer's instructions. To verify that secreted IL-1β in cell-culture supernatants was the 17-kDa bioactive form, we concentrated the supernatants using Amicon Ultra Centrifugal Filters (Millipore, Billerica, MA) according to the manufacturer's instructions, then performed immunoblot by which pro–IL-1β and bioactive IL-1β can be separated by size difference. The immunoblots showed only 17-kDa IL-1β, indicating that IL-1β in the supernatant is mostly bioactive IL-1β.

Confocal microscopy

Reactive oxygen species (ROS) was examined with a Zeiss LSM 510 confocal microscope. THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h, then treated with 10 μM CM-H_2DCFDA in prewarmed PBS for 30 min at 37˚C. Cells were washed three times with warm PBS, then treated with indicated concentration of sodium palmitate for 20 min in 0.25% FBS-RPMI 1640. Cells were then washed with ice-cold PBS, fixed in 10% formalin for 30 min at 4˚C, and washed again with ice-cold PBS. The cells were mounted on glass slides and analyzed with a 40 × 1.3 oil objective lens using laser excitation at 488 nm.

TLR2 small interfering RNA transfection assays

Silencer Select predesigned and prevalidated negative control and TLR2 small interfering RNAs (siRNAs; cat no. 4390843 and 4392420, respectively) were purchased from Life Technologies Corporation. THP-1 cells were transiently transfected with siRNAs (25 nM) using Lipofectamine RNAiMAX (catalog no. 13778) reagent following manufacturer's instructions. Cell-surface TLR2 expression was analyzed by flow cytometry 60 h after transfection; then cells were treated with sodium palmitate (150 μM) and Pam_3CSK_4 (10 ng/ml) for 24 h. Cell-culture supernatants were collected and analyzed for IL-1β by ELISA. For cell-surface expression of TLR2 by flow cytometry, transfected THP-1 cells were stained for 30 min on ice with anti-TLR2 Ab (clone TL2.1), then analyzed with a FACSCalibur flow cytometer by collecting 50,000 events in the FL1 channel.

TR-FRET assays to determine TLR2/1 dimerization

Anti-TLR2 (clone TL2.1, catalog no. 16-9922-82) and anti-TLR1 (clone GD2.F4, catalog no. 16-9911-82) Abs extensively characterized in traditional FRET experiments (33–36) were purchased from eBioscience (San Diego, CA) and custom labeled with europium cryptate (donor) and proprietary d2 dye (acceptor), respectively, by CisBio Bioassays (Bedford, MA). THP-1 cells (10^6 cells/ml) were serum-starved in 0.25% FBS-RPMI 1640 with 44.6 ng/ml TLR2-europium cryptate and 220 ng/ml TLR1-d2 Abs (donor/acceptor ratio of 1:5) for 12 h. Cells were washed in PBS to remove unbound Ab, then resuspended in 0.25% FBS-RPMI 1640. Cells were pretreated for 1 h if applicable, then treated for 10 min. Cells were washed in PBS, then fixed in 1% paraformaldehyde on ice for 20 min. Cells were washed again in PBS, then placed in incubation buffer (calcium- and magnesium-free DPBS with 400 mM potassium fluoride and 0.1% BSA) at a concentration of 3×10^6/ml. Fifty microliters per well was loaded into a half-area white 96-well plate and analyzed on a Synergy 2 plate reader (BioTek) configured with a 330/80 nm excitation filter and 620/10 and 665/8 emission filters. Fluorescence of the europium cryptate donor and d2 acceptor were measured, respectively, at 620 and 665 nm (100-μs time delay, 300-μs integration) upon 330 nm excitation. The 620- and 665-nm sample emissions were corrected for background by subtracting the fluorescence of incubation buffer at the respective wavelengths. The energy transfer ratio for each individual well was calculated using the formula: (signal 665 nm/signal 620 nm) × 10^4. Of the two sequential measurements carried out, the time resolved fluorescence emission measured at 620 nm is used as an internal reference correlated with cell-surface TLR2 expression levels. At the same time, the d2 emission measured at 665 nm upon 620-nm excitation signifies energy transfer through proximity of the labeled TLR1 and TLR2 Abs. The principle of TR-FRET is illustrated in Fig. 5C. All experimental samples were run in triplicate.

Isolation of lipid raft fractions using sucrose gradient ultracentrifugation

THP-1 cells (5×10^7) stimulated with palmitic acid and Pam_3CSK_4 were used to isolate lipid rafts. Cells were incubated in serum-poor RPMI 1640 (1.0% FBS) for 12 h before treatment. Lipid rafts were isolated by lysing cells in 750 μl TNE lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 5 mM EDTA, with 1% Triton X-100, 1 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM PMSF, and 10 μg/ml each of aprotinin and leupeptin) for 30 min on ice followed by passage through a 25-gauge needle. Lysates were mixed with an equal volume of 80% sucrose in TNE buffer and placed in an ultracentrifuge tube. Two milliliters of 30% sucrose in TNE buffer was then overlaid followed by 1 ml 5% sucrose in TNE buffer. Centrifuge tubes were placed in an SW60 rotor in a Beckman L-70 Ultracentrifuge and spun for 16 h at 40,000 rpm at 4˚C using maximum acceleration and no brake conditions. After fractionation, eight 500-μl fractions were collected from top down. For immunoblotting, fractionated samples were mixed with sample buffer and subjected to electrophoresis.

Preparation of triglyceride-rich lipoproteins and triglyceride-rich lipoprotein lipolysis products

Postprandial blood samples were obtained from healthy male and female volunteers 3.5 h after consumption of a standard moderately high-fat meal (40% calories from fat). This time point has been shown to correlate with elevated circulating triglyceride-rich lipoproteins (TGRLs) (37, 38). All procedures were conducted under a protocol approved by the Human Subjects Institutional Review Committee at the University of California Davis. Written informed consent was obtained from all study subjects before participating. Blood was collected in 10-ml Vacutainer tubes containing K_2-EDTA (BD Biosciences). TGRL isolated as previously described (39–41) contained ≤0.5 endotoxin unit/ml according to a Pyrochrome kit from Associates of Cape Cod (Falmouth, MA). Samples were stored over nitrogen gas in sealed tubes at 4˚C. For preparation of TGRL lipolysis products, TGRL (50 mg/dl triglycerides) was subjected to enzymatic lipolysis with bovine LPL (5 U/ml) in 0.25% (for THP-1 cell treatments) or 2% (for primary monocyte treatments) HI-FBS RPMI 1640 for 60 min at 37˚C. The conditioned media were immediately used for incubations with cells in culture for indicated times.

Isolation of primary human monocytes and treatment with fatty acids

Whole blood was collected in 10-ml Vacutainer tubes containing sodium heparin (BD Biosciences). Blood was centrifuged at 1400 × *g* for 10 min at room temperature. Plasma was removed and buffy coats were collected, mixed with an equal volume of HBSS, then overlaid on an equal volume of Histopaque-1077 (Sigma) and centrifuged at 400 × g for 30 min. PBMCs were isolated, washed twice with HBSS, then resuspended at 1×10^6 monocytes/ml in serum-free RPMI 1640. After 2 h at 37˚C in a 5% CO_2/air environment, the medium containing nonadherent cells was aspirated, the attached monocytes were washed twice with HBSS to remove residual nonadherent cells, and then cultured in HI-FBS RPMI 1640. Monocytes were then treated with palmitic acid or TGRL lipolysis products for 24 h. Supernatants were collected and analyzed for IL-1β by ELISA.

Treatment of whole blood with LPL

Blood from healthy male and female volunteers, as approved by the Human Subjects Institutional Review Committee of the University of California Davis, was drawn and prepared at the Western Human Nutrition Research Center-U.S. Department of Agriculture to study whether endogenous SFAs derived from enzymatic hydrolysis of TGRLs can induce inflammasome-mediated IL-1β release. Written, informed consent was obtained from all study subjects before participating. Fasting and 3.5-h postprandial whole blood after consumption of a 630-calorie high-fat breakfast containing 40% total fat (20% saturated fat), 16% protein, and 44% carbohydrate was collected in a 10-ml Vacutainer tube containing sodium heparin (BD Biosciences) and then diluted 1:1 with serum-free RPMI 1640. If necessary, RPMI 1640 diluted whole blood was pretreated with DHA (10 μM) for 1 h, then subjected to enzymatic lipolysis with bovine LPL (4 U/ml) for 24 h at 37˚C in a 5% CO_2/air environment. Supernatants were collected and analyzed for IL-1β by ELISA.

Statistical analyses

One-way ANOVA was used to determine significance of TR-FRET and IL-1β concentration differences in cell-culture supernatants. Tukey's multiple-comparison test was used as a posttest if any differences were noted with ANOVA. Differences in IL-1β concentrations in whole blood assays were determined using two-way repeated-measures ANOVA. Factors included metabolic state (fasting and postprandial) and treatment (control, LPL, and LPL + DHA), and the interaction of metabolic state × treatment. Post hoc comparison was accomplished with Bonferroni posttests. Differences within each metabolic state of the whole blood assays were determined using repeated-measures ANOVA followed by Tukey's multiple-comparison test (GraphPad Software, La Jolla, CA). A p value <0.05 was considered statistically significant.

Results

Palmitic acid induces the expression of pro–IL-1β and secretion of mature IL-1β in THP-1 monocytes

Sodium palmitate treatment led to the expression of pro–IL-1β in a time- and dose-dependent manner in THP-1 cells, whereas untreated cells showed undetectable levels of pro–IL-1β (Fig. 1A, 1C). Sodium palmitate also induced secretion of mature IL-1β in cell-culture supernatant (Fig. 1B, 1C), which requires caspase-1 for the cleavage of pro–IL-1β. BSA-solubilized palmitic acid also induced secretion of mature IL-1β in the supernatant in a dose-dependent manner (Supplemental Fig. 1A). Inhibition of caspase-1 activity by Ac-YVAD-AOM reduced sodium palmitate–mediated secretion of IL-1β (Fig. 1D). Untreated THP-1 monocytes contain both procaspase-1 and inflammasome-mediated active caspase-1 (Fig. 1A). These results suggest that unstimulated monocytes possess constitutively active inflammasome. Therefore, primary signals inducing the expression of pro–IL-1β mediated by the activation of TLRs are sufficient for the production of IL-1β in monocytes. In this respect, monocytes differ from macrophages, which do not contain constitutively active inflammasome, and thus require the secondary signals for the activation of inflammasome and the secretion of mature IL-1β (9–11). Untreated THP-1 cells also express NALP3 (Fig. 1A), which was enhanced by sodium palmitate treatment (Supplemental Fig. 2), suggesting that palmitate not only provides the primary signal leading to the expression of pro–IL-1β, but also potentiates the activation of inflammasome by upregulating the expression of NALP3.

DHA inhibits palmitic acid–induced expression of pro–IL-1β and consequent release of mature IL-1β in monocytes

Our previous studies showed that DHA is a pan inhibitor for TLR activation (20, 21, 23–25). Therefore, we determined whether the n-3 polyunsaturated fatty acid (PUFA) DHA inhibits palmitic acid–induced expression of pro–IL-1β and secretion of mature IL-1β. Pretreating THP-1 cells with DHA dose-dependently inhibited the expression of pro–IL-1β after a 6-h treatment with sodium palmitate (150 μM; Fig. 2A) and the secretion of active IL-1β and caspase-1 after a 24-h treatment (Fig. 2B). Pretreating THP-1 cells with DHA also dose-dependently inhibited the secretion of mature IL-1β after a 24-h treatment with BSA-solubilized palmitic acid (Supplemental Fig. 1B). These results imply that DHA inhibits palmitic acid–induced activation of TLRs (primary signal) and the subsequent expression of pro–IL-1β.

Palmitic acid induces NF-κB activation through TLR2 dimerized with TLR1 or TLR6 and through TLR4, and activates downstream signaling pathways of PRRs

Pro–IL-1β is one of the TLR target gene products. To determine whether palmitic acid–induced expression of pro–IL-1β is mediated through activation of TLRs, we transfected cells with expression plasmids of TLRs known to recognize ligands containing saturated lipid chains. HEK293T cells cotransfected with TLR2 and TLR1, TLR2 and TLR6, and TLR4 and MD-2 in addition to NF-κB–luciferase reporter and β-galactosidase expression vectors were treated with BSA-solubilized palmitic acid (C16:0-BSA; Fig. 3A–C). BSA-solubilized palmitic acid dose-dependently induced NF-κB activation through all transfected TLRs indicating palmitic acid–induced pro–IL-1β is at least in part mediated through activation of TLR2 and TLR4. Treatment of THP-1 cells with sodium palmitate activated the downstream signaling pathways of TLRs, leading to enhanced phosphorylation of JNK and IκBα (Fig. 3D).

Pam_3CSK_4 induces, but DHA inhibits, the expression of pro–IL-1β and secretion of mature IL-1β in THP-1 monocytes

To confirm that pro–IL-1β expression and IL-1β secretion in THP-1 monocytes are at least in part mediated through activation of TLR2 and TLR1, we stimulated cells with the TLR2/1-specific

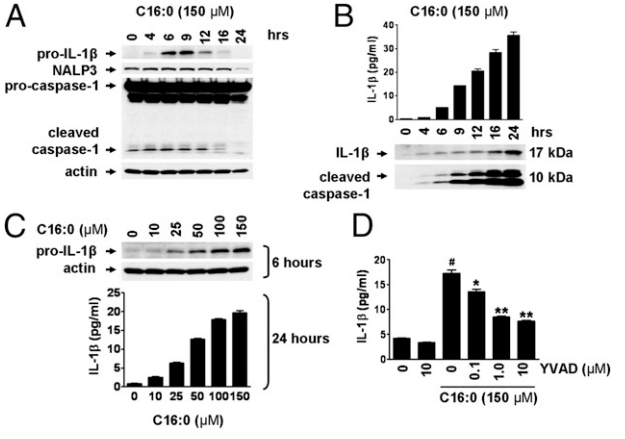

FIGURE 1. Palmitic acid induces pro–IL-1β expression and inflammasome-mediated IL-1β secretion in THP-1 monocytes. (**A** and **B**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h, then treated with C16:0 (150 μM) for the indicated times. (A) Cell lysates were immunoblotted for pro–IL-1β, NALP3, and caspase-1. (B) IL-1β in cell-culture supernatant was analyzed by ELISA and immunoblot. Cleaved caspase-1 in supernatant was analyzed by immunoblot. (**C**) Serum-starved THP-1 cells were treated with indicated concentrations of C16:0. After 6 h, cell lysates were immunoblotted for pro–IL-1β. After 24 h, cell-culture supernatants were analyzed for IL-1β by ELISA. Data in (B) and (C) are expressed as mean ± SD and are representative of three independent experiments with similar results. (**D**) Serum-starved THP-1 cells were incubated with caspase-1 inhibitor (Ac-YVAD-AOM) for 1 h, then treated with C16:0 for 24 h. IL-1β in supernatant was analyzed by ELISA. Data are expressed as mean ± SEM of three independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $^{*}p < 0.05$, $^{**}p < 0.01$ significantly different from C16:0).

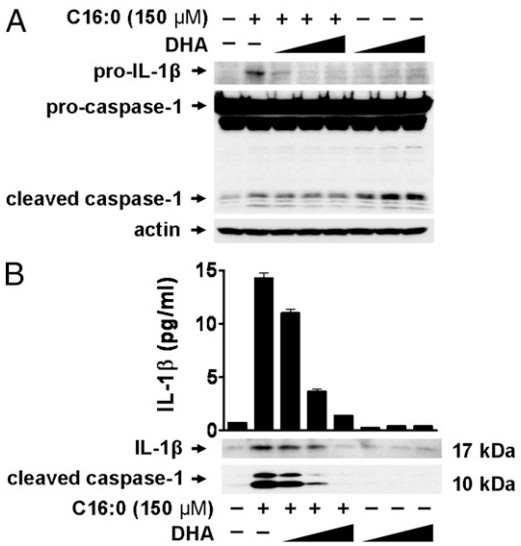

FIGURE 2. Palmitic acid induces, but DHA inhibits, the expression of pro–IL-1β and inflammasome-mediated IL-1β production. (**A** and **B**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h. Cells were incubated with DHA (2.5, 5, and 10 μM) for 1 h, then treated with C16:0 (150 μM). (A) After 6 h, cell lysates were immunoblotted for pro–IL-1β and caspase-1. (B) After 24 h, cell-culture supernatants were analyzed for IL-1β by immunoblot and ELISA. Secreted caspase-1 was analyzed by immunoblot. Data are expressed as mean ± SD and are representative of three independent experiments with similar results.

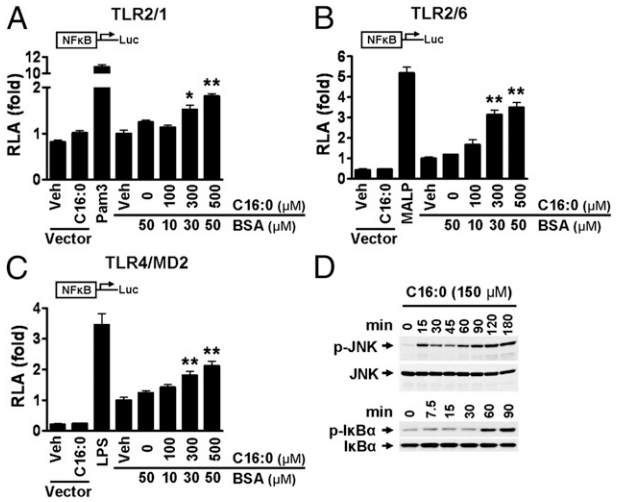

FIGURE 3. Palmitic acid induces NF-κB activation through TLR2 dimerized with TLR1 or TLR6 and through TLR4, and activates the downstream signaling pathways of PRRs. (**A–C**) HEK293T cells were cultured in 10% FBS/DMEM medium and cotransfected with TLR2 and TLR1 (A), TLR2 and TLR6 (B), or TLR4 and MD2 (C) in addition to NF-κB–luciferase reporter and β-galactosidase expression vectors. After 24 h, the cells were serum-starved in 0.25% FBS/DMEM for 6 h and then treated with C16:0-BSA for 12 h. The cell lysates were assayed for luciferase and galactosidase activities. Values are expressed as relative luciferase activity (RLA). Controls: Pam3 (Pam_3CSK_4, TLR2/1 agonist, 10 ng/ml), MALP (MALP-2, TLR2/6 agonist, 10 ng/ml), LPS (TLR4 agonist, 50 ng/ml). pDisplay empty vector was used as a negative control. Data are expressed as mean ± SEM of three independent experiments. Significance was determined by unpaired, two-tailed *t* test ($*p < 0.05$, $**p < 0.01$ significantly different from 50 μM BSA). (**D**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h. Cells were treated with C16:0 (150 μM) for the indicated times, and cell lysates were immunoblotted for phosphorylated JNK, total JNK, phosphorylated IκBα, and total IκBα.

triacylated lipopeptide ligand Pam_3CSK_4 (42). Treatment led to expression of pro–IL-1β in a time-dependent manner and secretion of IL-1β in a dose-dependent manner (Fig. 4A, 4B). Inhibition of caspase-1 activity by Ac-YVAD-AOM reduced Pam_3CSK_4-mediated secretion of IL-1β (Fig. 4C). Pretreating THP-1 cells with DHA dose-dependently inhibited the secretion of mature IL-1β after a 24-h treatment with Pam_3CSK_4 (Fig. 4D). Activation of TLR2 has been shown to induce apoptosis in mononuclear phagocytes, including in THP-1 cells (43, 44). Activation of inflammasome and caspase-1 also induces pyroptotic cell death (45). Therefore, we compared the cell death induced by Pam_3CSK_4 with that by sodium palmitate in THP-1 cells. Both treatments induced comparable levels of cell death in a time-dependent manner exhibiting a similar cellular response (Supplemental Fig. 3A, 3B).

Palmitic acid–induced IL-1β secretion is mediated, at least in part, through activation of TLR2

Because TLR2 is predominantly expressed compared with TLR4 in monocytes as determined by flow cytometry (data not shown) and RT-PCR (26), we determined whether knocking down TLR2 expression by siRNAs suppresses palmitic acid–induced IL-1β secretion in THP-1 cells. Our results showed that reducing cell-surface expression of TLR2 with siRNA (Fig. 5B) suppressed Pam_3CSK_4- and sodium palmitate–induced IL-1β 63 and 15%, respectively (Fig. 5A). Because TLR2 siRNA modestly inhibited palmitic acid–induced IL-1β, we questioned to what extent palmitic acid–induced IL-1β might be mediated through TLR4. Thus, we used the TLR4-specific small-molecule inhibitor TAK-

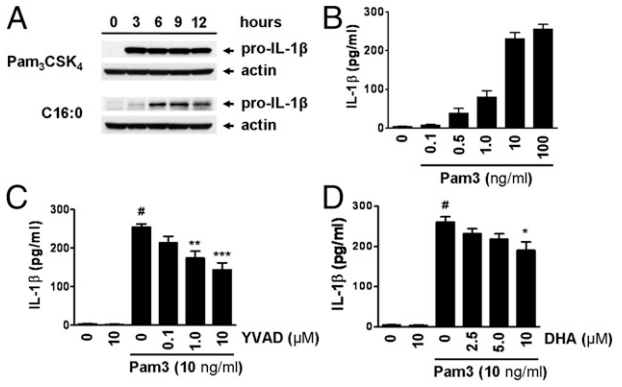

FIGURE 4. Pam_3CSK_4 induces pro–IL-1β expression and inflammasome-mediated IL-1β secretion in THP-1 monocytes. (**A**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h, then treated with Pam_3CSK_4 (10 ng/ml) and C16:0 (150 μM) for the indicated times. Cell lysates were immunoblotted for pro–IL-1β. (**B**) Serum-starved THP-1 cells were treated with Pam_3CSK_4 at indicated concentrations for 24 h. Cell-culture supernatants were analyzed for IL-1β by ELISA. Data in (A) and (B) are expressed as mean ± SD and are representative of two independent experiments with similar results. (**C** and **D**) Serum-starved THP-1 cells were incubated with caspase-1 inhibitor (Ac-YVAD-AOM) (C) or DHA (D) for 1 h, then treated with Pam_3CSK_4 for 24 h. Cell-culture supernatants were analyzed for IL-1β by ELISA. Data in (C) and (D) are expressed as mean ± SEM of three independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ significantly different from Pam_3CSK_4).

242 in combination with LPS, sodium palmitate, and non-TLR4 ligands that activate PRRs, which are known to be induced by SFAs. Our results showed that TAK-242 inhibited LPS-induced IL-1β by 80% but inhibited sodium palmitate–induced IL-1β by only 19% (Supplemental Fig. 4). Because SFAs are also known to induce proinflammatory signaling pathways through activation of Nods (26) and the unfolded protein response (46), inhibiting both TLR2 and TLR4 is unlikely to abolish palmitic acid–induced IL-1β secretion. Therefore, as an alternate to the siRNA approach and to determine the specificity of palmitic acid for activating TLR2, we used TR-FRET assays. Receptor dimerization is known as the proximal event in the activation of TLRs (42, 47–49). Therefore, we determined whether palmitic acid directly induces dimerization of endogenous TLR2/1. We used an anti-TLR2 Ab labeled with a lanthanide cryptate fluorophore with a long-lived emission (1 ms) as an energy donor, and an anti-TLR1 Ab labeled with an acceptor fluorophore to measure energy transfer through proximity of labeled TLR1 and TLR2 (Fig. 5C). Our results showed that both sodium palmitate and Pam_3CSK_4 induced, but DHA inhibited, dimerization of TLR2 with TLR1 as determined by TR-FRET (Fig. 5D). These results further support that TLR2 is, at least in part, responsible for palmitic acid–induced IL-1β production.

Palmitic acid induces the translocation of MyD88 and p47^{phox} (organizer protein of NADPH oxidase 2) into lipid rafts, whereas DHA inhibits palmitic acid–induced translocation of MyD88 and p47^{phox}

Receptor dimerization and translocation of membrane TLRs and adaptor proteins to lipid rafts upon agonist stimulation are the proximal events required for activation of downstream signaling pathways (25, 34–36, 50–55). Our previous studies showed that LPS or lauric acid induces dimerization and translocation of TLR4 into lipid rafts where it recruits downstream signaling molecules including an adaptor molecule MyD88 in an ROS-dependent manner (25). Because TLR2 activation also requires transloca-

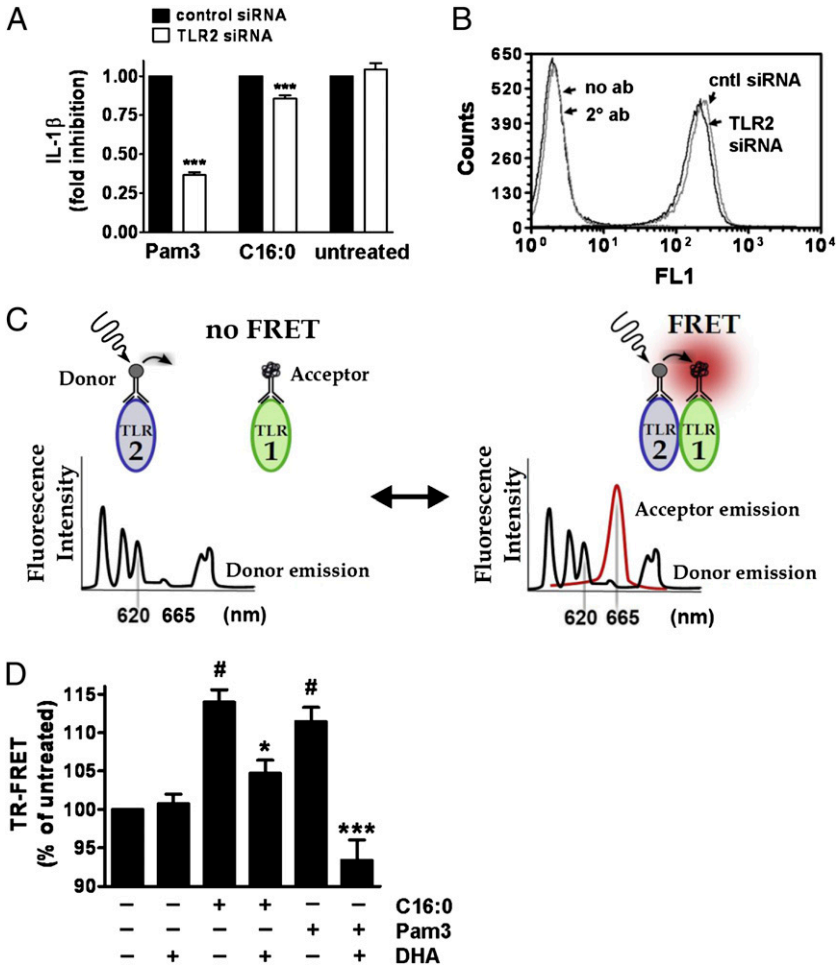

FIGURE 5. Palmitic acid–induced IL-1β secretion in THP-1 cells is mediated through activation of TLR2. (**A**) THP-1 cells were transfected with negative control or TLR2 siRNA. After 48 h, cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h, then treated with C16:0 (150 μM) or Pam_3CSK_4 (10 ng/ml) for 24 h. Supernatants were analyzed for IL-1β by ELISA. Data are presented as mean percentages of control siRNA-treated cells ± SEM and were calculated from three independent experiments. $***p < 0.001$. Significance was determined by two-tailed, unpaired *t* test. (**B**) TLR2 cell-surface expression on THP-1 cells was measured 60 h after transient transfection with siRNAs by flow cytometry. Data are representative of at least three independent experiments. (**C**) Schematic of the TR-FRET assays performed to detect dimerization of TLR2 with TLR1 using anti-TLR2 Abs labeled with europium cryptate as the donor fluorophore and anti-TLR1 Abs labeled with d2 as the acceptor fluorophore. (**D**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h with TLR2 and TLR1 Abs, then incubated with DHA (10 μM) for 1 h and treated with C16:0 (150 μM) or Pam_3CSK_4 (100 ng/ml) for 10 min. TR-FRET data are presented as mean percentages of untreated cells ± SEM and were calculated from at least five independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $*p < 0.05$, $***p < 0.001$ significantly different from C16:0 and Pam3, respectively).

tion and association of the adaptor molecule MyD88 and NADPH oxidase 2 (NOX2) into lipid rafts (51, 56, 57), we determined whether palmitic acid induces the translocation of TLR2, MyD88, and NOX2 into lipid rafts of THP-1 cells by isolating lipid raft fractions and examining the translocation of these proteins by immunoblotting. Our results showed that flotillin-1, which is constitutively expressed in lipid rafts (58, 59), localized to fractions 2 and 3, whereas transferrin receptor, which is constitutively expressed in nonlipid rafts (58), localized to fractions 6–8 (Fig. 6A). Although the majority of TLR2 resided in nonlipid raft fractions, a substantial amount was expressed in lipid raft fractions isolated from unstimulated cells (Fig. 6A). Within 5 min of treatment, both sodium palmitate and Pam_3CSK_4 induced translocation of MyD88 and NOX2 organizer protein $p47^{phox}$ to lipid raft fractions in THP-1 monocytes (Fig. 6B). In contrast, DHA inhibited both sodium palmitate– and Pam_3CSK_4-induced recruitment of both MyD88 and $p47^{phox}$ to lipid raft fractions (Fig. 6C).

TLR2-mediated palmitic acid–induced IL-1β is dependent on NADPH oxidase activation and is inhibited by DHA

Based on our results indicating that palmitic acid induces the assembly of NOX2 through translocation of $p47^{phox}$ to lipid rafts, we determined whether palmitic acid–induced assembly of NOX2 would induce ROS. Treatment with sodium palmitate induced ROS in a dose-dependent manner as assessed by confocal microscopy but was inhibited by DHA (Fig. 7A). Because NOX2 is known to be a critical component of the TLR2 signaling complex (56, 57, 60) we determined whether inhibiting NOX2 assembly would inhibit palmitic acid–induced IL-1β secretion. Apocynin, which inhibits the assembly and activation of NOX2 by interfering with the translocation of $p47^{phox}$ (61, 62) dose-dependently inhibited sodium palmitate– and Pam_3CSK-induced IL-1β secretion (Fig. 7B). In addition, apocynin dose-dependently inhibited sodium palmitate–induced dimerization of TLR2/1 as assessed by TR-FRET (Fig. 7C). Collectively, these results reveal that palmitic acid and DHA reciprocally modulate activation of TLR2 by modulating the dimerization of TLR2/1, and that dimerization is dependent on NOX2 activation.

Both exogenous palmitic acid and endogenous fatty acids derived from the lipolysis products of TGRLs induce, but DHA inhibits, IL-1β production in human peripheral blood monocytes and whole blood

Next, we determined whether palmitic acid also induces IL-1β production in primary human monocytes. Sodium palmitate induced, but DHA inhibited, IL-1β production in primary monocytes cultured in 1% HI-FBS RPMI 1640 (Fig. 8A) similar to the results obtained from the THP-1 monocytic cell line. Next, we determined whether a physiological source of dietary SFAs recapitulates the effects of palmitic acid. Postprandial lipemia is characterized by transient accumulation of TGRLs in the blood after ingestion of a fatty meal. In vivo, TGRLs are hydrolyzed by LPL, an enzyme anchored to endothelial cells, releasing free fatty acids into the blood in immediate proximity to blood monocytes. Our previous studies showed that palmitic acid is the major SFA found in the lipolysis products (free fatty acids) of these TGRLs isolated from subjects consuming the high-fat meal (40). There-

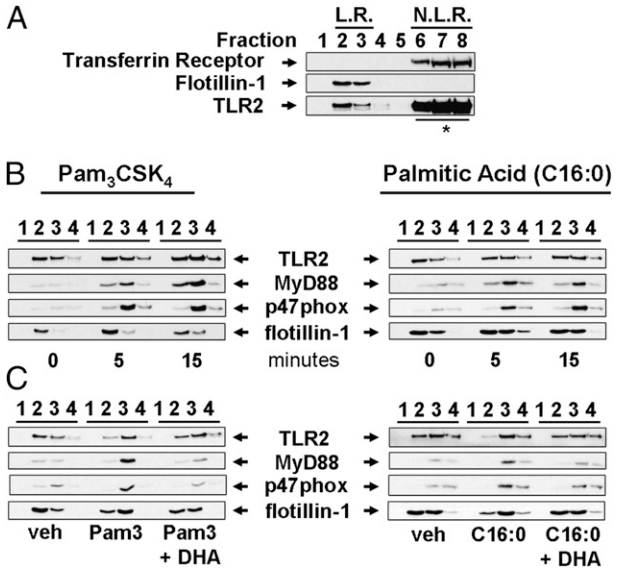

FIGURE 6. Palmitic acid and Pam_3CSK_4 induce, but DHA inhibits, the recruitment of MyD88 and $p47^{phox}$ (subunit of NOX2) into lipid raft (LR) fractions. (**A**) Cells were lysed and fractionated by sucrose-gradient ultracentrifugation to demonstrate the separation of LR and nonlipid raft (NLR) fractions of plasma membrane. LR fractions (fractions 2 and 3) and NLR fractions (fractions 6–8) were identified by the presence of flotillin-1 (LR marker) and transferrin receptor (NLR marker), respectively. Asterisk denotes because of overwhelming expression, only 20% of input lysate from fractions 6–8 was subjected to SDS-PAGE and immunoblotted with anti-TLR2 Abs. (**B**) THP-1 cells were serum-starved in 1.0% FBS-RPMI 1640 for 12 h, then treated with C16:0 (150 μM) or Pam_3CSK_4 (100 ng/ml) for indicated time periods to determine whether palmitic acid (C16:0) or Pam_3CSK_4 induces recruitment of the downstream signaling components of TLR2 into LR fractions. Fractions 1–4 were immunoblotted with anti-TLR2, anti-MyD88, anti-$p47^{phox}$, and anti–flotillin-1 Abs. (**C**) Serum-starved THP-1 cells were incubated with DHA (10 μM) for 1 h, then treated with C16:0 (150 μM) or Pam_3CSK_4 (100 ng/ml) for 5 min. Cell lysate was separated by sucrose-gradient ultracentrifugation, and fractions were immunoblotted.

fore, we determined whether the lipolysis products of TGRLs also induce IL-1β production in primary monocytes or in whole blood. The lipolysis products of TGRLs induced IL-1β production in primary monocytes (Fig. 8B). Increased IL-1β production by the treatment of primary monocytes with conditioned media prepared with LPL without added TGRL is likely due to release of free fatty acids from lipids present in FBS (2% final concentration in media). Lipolysis products of TGRLs also increased heterodimerization of TLR2 with TLR1 as assessed by TR-FRET in THP-1 monocytes treated in 0.25% FBS (Fig. 8C). Finally, direct treatment of fasting and postprandial whole blood with LPL induced robust production of IL-1β. Treatment of postprandial whole blood with LPL induced significantly more IL-1β compared with fasting whole blood. Furthermore, concomitant treatment of both LPL-treated fasting and postprandial whole-blood samples with DHA inhibited the IL-1β production (Fig. 8D). These results suggest that inflammasome-mediated IL-1β production in blood monocytes can be dynamically modulated by the types of dietary fat we consume (Fig. 9).

Discussion

Our results demonstrate that palmitic acid, a predominant dietary SFA, induces, whereas the n-3 fatty acid DHA inhibits, the expression of pro–IL-1β and subsequent release of mature IL-1β in both THP-1 monocytes and primary human blood monocytes. These results further support the findings that blood monocytes contain active caspase-1 because of the presence of constitutively active NALP3 inflammasome (8–10). Therefore, primary signals inducing the expression of pro–IL-1β are sufficient for inflammasome-mediated IL-1β production in blood monocytes. Pro–IL-1β is one of the target gene products derived from the activation of TLRs expressed in monocytes. Therefore, any agonists that activate TLRs should be able to induce inflammasome-mediated IL-1β production in human blood monocytes without secondary signals required for the activation of inflammasome as depicted in Fig. 9.

Animal studies showed that palmitic acid can activate NALP3 inflammasome in bone marrow–derived macrophages (BMDMs) pretreated with LPS; however, palmitic acid alone did not induce pro–IL-1β expression in BMDMs, suggesting that palmitic acid can provide the secondary signal to activate inflammasome, but not the primary signals to induce the expression of pro–IL-1β in BMDMs (19). Thus, for palmitic acid to induce inflammasome-

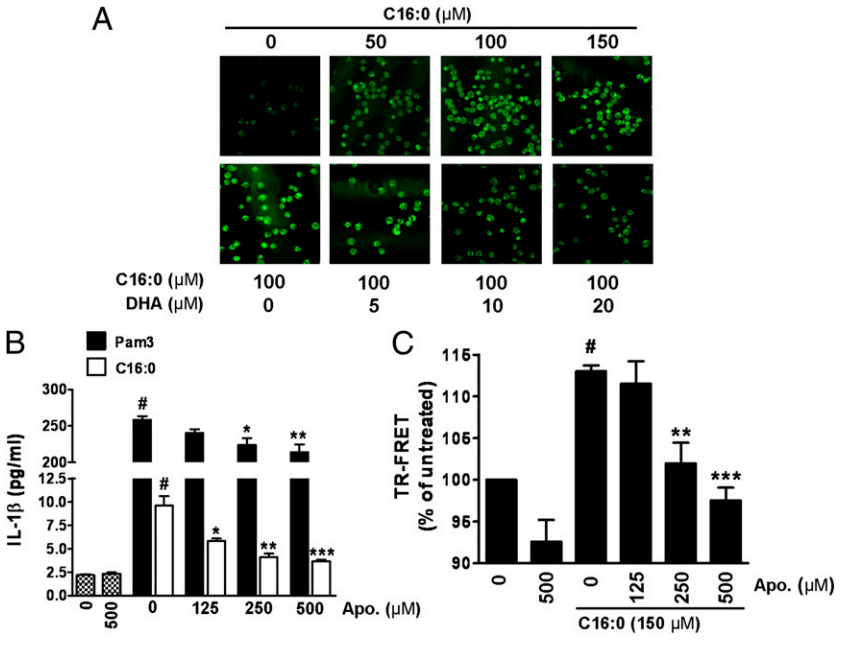

FIGURE 7. Palmitic acid induces, but DHA inhibits, ROS production. (**A**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h. Cells were incubated with CM-H2DCFDA (10 μM) for 30 min, then pretreated with or without DHA for 1 h before treatment with C16:0 for 20 min. Cells were fixed and imaged by confocal microscopy. Original magnification ×40. (**B**) Serum-starved THP-1 cells were incubated with apocynin for 1 h, then treated with C16:0 (150 μM) or Pam_3CSK_4 (10 ng/ml) for 24 h. Cell-culture supernatants were analyzed for IL-1β by ELISA. Data are expressed as mean ± SEM of three independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from C16:0 or Pam3). (**C**) THP-1 cells were serum-starved with fluorophore-labeled TLR2 and TLR1 Abs for 12 h. Cells were incubated with apocynin (125, 250, 500 μM) for 1 h, then treated with C16:0 (150 μM) for 10 min. TR-FRET data are presented as mean percentages of untreated cells ± SEM and were calculated from at least three independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from C16:0).

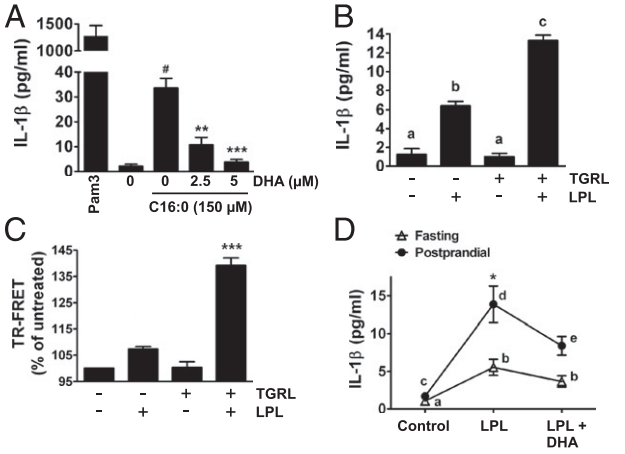

FIGURE 8. Palmitic acid and endogenous fatty acids derived from the lipolysis products of TGRLs induce, but DHA inhibits, IL-1β production in human blood monocytes or whole blood. (**A**) Primary monocytes cultured in 0.25% HI-FBS–RPMI 1640 were incubated with or without DHA for 1 h, then treated with C16:0 (150 μM) for 24 h. Cell-culture supernatants were analyzed for IL-1β by ELISA. Data are expressed as mean ± SEM of three independent experiments. Significance was determined by ANOVA ($^{\#}p < 0.001$ significantly different from untreated, $^{**}p < 0.01$, $^{***}p < 0.001$ significantly different from C16:0). Pam3 (50 ng/ml) was used as a positive control. (**B**) Primary monocytes cultured in 2% HI-FBS–RPMI 1640 were treated with LPL-, TGRL-, or LPL + TGRL-conditioned media for 24 h, and supernatants were analyzed for IL-1β by ELISA. Bars not sharing a common superscript are significantly different ($p < 0.05$) as determined by ANOVA. (**C**) THP-1 cells were serum-starved in 0.25% FBS-RPMI 1640 for 12 h with fluorophore-labeled TLR2 and TLR1 Abs, then treated with LPL-, TGRL-, or LPL + TGRL-conditioned media for 10 min. TR-FRET data are presented as mean percentages of untreated cells ± SEM and were calculated from four independent experiments. Significance was determined by ANOVA ($^{***}p < 0.001$ significantly different from all treatments). (**D**) Fasting and postprandial whole blood was incubated with DHA (10 μM) for 1 h, then treated with LPL for 24 h. Supernatants were analyzed for IL-1β by ELISA. Data are presented as mean ± SEM. Two-way repeated-measures ANOVA was performed to test for the effects of metabolic state, treatment, and metabolic state × treatment. There was a significant effect of metabolic state ($p = 0.0001$), treatment ($p = 0.0002$), and metabolic state × treatment ($p = 0.0079$). Means for each of the three treatments were compared by Bonferroni posttests. Fasting LPL was significantly different from postprandial LPL ($^{*}p < 0.001$). Within each metabolic state, points with different superscripts are significantly different ($p < 0.05$); $n = 12$ for fasting and $n = 15$ for postprandial.

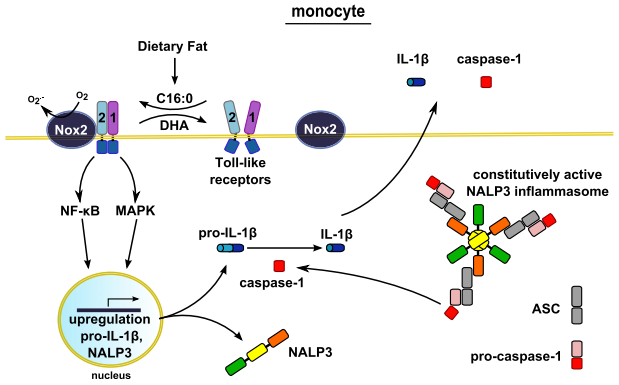

FIGURE 9. Illustration depicting the modulation of the production of NALP3 inflammasome-mediated IL-1β in human monocytes by dietary fatty acids. The major dietary SFA palmitic acid (C16:0) induces, but the n-3 fatty acid DHA inhibits, heterodimerization of TLR2 with TLR1. The heterodimerization leads to the activation of downstream intracellular signaling pathways that culminates in the expression of pro–IL-1β, which is cleaved by constitutively activated caspase-1 to secrete mature IL-1β. Therefore, the primary signals to induce the expression of pro–IL-1β are sufficient for the production of mature IL-1β in human monocytes. Palmitic acid also upregulates the expression of NALP3, which could potentiate NALP3 inflammasome activity.

mediated IL-1β secretion in macrophages, the expression of pro–IL-1β by the primary signals (e.g., LPS) would be required. In contrast with BMDMs, bone marrow–derived DCs (BMDCs) were shown to secrete inflammasome-mediated IL-1β upon TLR activation by free fatty acids or LPS alone, suggesting that the primary signal is sufficient to induce NALP3 inflammasome-mediated IL-1β secretion in BMDCs (3, 11). The different ability of mononuclear phagocytes to secrete NALP3 inflammasome-mediated IL-1β may be explained, at least in part, by the differential levels of expression of TLRs and NALP3 in steady-state conditions. Monocytes constitutively express NALP3 and active caspase-1 in steady-state conditions (Fig. 1A). NALP3 is detectable in unstimulated BMDCs, but not BMDMs (11). Furthermore, BMDCs were shown to express more TLR2 and TLR4 compared with BMDMs (3). Consequently, BMDCs expressed higher amounts of both pro–IL-1β and NALP3 proteins in response to TLR ligands than did BMDMs (11). Therefore, the inability of palmitic acid alone to induce NALP3 inflammasome-mediated IL-1β production in macrophages may be because of the lower expression levels of TLR2 and TLR4 compared with monocytes or DCs. As a result, palmitic acid may be unable to sufficiently activate TLR signaling pathways to induce adequate levels of pro–IL-1β and NALP3 expression despite the ability of palmitic acid to provide the secondary signals activating the inflammasome.

How SFAs can provide the secondary signals for inflammasome activation is an intriguing question. A recent animal study showed that C2 ceramide can activate NALP3 inflammasome in BMDMs (18). Another animal study showed that high-fat diet stimulates ceramide synthesis in a TLR4-dependent manner and suggested that ceramide mediates high-fat diet–induced insulin resistance (14). These results suggest that the degree to which fatty acids modulate NALP3 inflammasome-mediated IL-1β production is cell type specific. Therefore, SFAs derived from high-fat diet in vivo can render both primary and secondary signals in tissues containing infiltrating monocytes, DCs, and macrophages through activation of TLRs leading to the expression of pro–IL-1β and by stimulating synthesis of endogenous molecules (e.g., ceramide) that can activate the inflammasome.

Next, we determined whether endogenous SFAs derived from the high-fat diet also induce inflammasome-mediated IL-1β release in human blood monocytes. Upon digestion and absorption, dietary SFAs are incorporated primarily into TGRLs. Our previous human studies showed that palmitic acid is the major SFA released after the treatment of postprandial TGRL with LPL (40). Therefore, we determined whether the lipolysis products of TGRL containing dietary palmitic acid can also induce inflammasome-mediated IL-1β. Our results showed that lipolysis products of TGRL isolated from postprandial blood derived from human subjects consuming the high-fat meal are sufficient to induce inflammasome-mediated IL-1β production in blood monocytes. Treatment of whole-blood samples with LPL also induced IL-1β release; IL-1β release from the postprandial whole blood was greater than that of fasting whole blood. LPL-induced IL-1β secretion in both fasting and postprandial whole blood was suppressed by DHA. Together, these results are significant in view of the fact that blood monocytes are sentinel immune cells in constant surveillance of invading pathogens, tissue injury, and metabolic fluctuations. Our

results suggest that the propensity of monocyte activation in response to such stimuli can be greatly modulated by types of plasma fatty acids, which, in turn, are altered by types of dietary fat consumed.

How SFAs induce, but DHA inhibits, TLR activation that leads to pro–IL-1β expression is an important question. Both TLR4 and TLR2 ligands LPS and triacylated lipopeptide Pam_3CSK_4, respectively, are acylated by SFAs. No ligands for TLRs other than TLR4 and TLR2 so far identified are known to be acylated by fatty acids. If these SFAs are removed from LPS or Pam_3CSK_4, the ligands completely lose ability to activate their respective receptor, demonstrating that these SFAs are required for their ligand activity (48, 63–68). Lipid A acylated by unsaturated fatty acids instead of SFAs is nontoxic and acts as an antagonist against the wild type endotoxin (64, 69, 70). Indeed, many studies have suggested that SFAs, but not unsaturated fatty acids, can activate TLR4 and TLR2 in both in vitro and in vivo systems (3, 16, 20, 21, 23, 24, 30, 31, 71–77). However, direct evidence that palmitic acid can activate TLRs has not been reported.

The X-ray crystallographic structure for the TLR2-TLR1 heterodimer revealed that two ester-bound fatty acyl (palmitic acid) chains of Pam_3CSK_4 are inserted into the hydrophobic lipid binding pocket in TLR2, whereas the amide-bound fatty acyl group is inserted into a hydrophobic channel in TLR1 (42). Thus, binding of fatty acyl groups of Pam_3CSK_4 into TLR2 induces heterodimerization of TLR2 with TLR1. Structure-function analysis for the lipopeptides revealed that the two ester-bound fatty acids are an essential determinant for its ligand activity for TLR2, and that the acyl chain with 16 carbons (palmitic acid) provides optimal stimulatory activity compared with ester-bound fatty acyl chains of different length (63). It is an interesting question whether palmitic acid itself without the peptide moiety can interact with the hydrophobic lipid binding sites in TLR2 or TLR1 and promote the dimerization of the receptors leading to recruitment of the immediate downstream signaling molecules including MyD88 and NOX2. Although the activation of downstream signaling molecules such as NF-κB or target gene expression are often used as surrogate markers of TLR activation, they do not necessarily reflect direct activation of TLRs because many receptors other than TLRs also induce NF-κB activation and similar target gene expression. Dimerization of TLRs is the most proximal event for the receptor activation required for the activation of downstream signaling pathways (25, 35, 51, 54, 78). Therefore, dimerization of TLRs would be a faithful readout for the direct activation of TLRs. So far, no direct evidence that palmitic acid can induce TLR dimerization has ever been presented.

Dimerization allows for the proper orientation of the TIR domains of TLRs to induce the recruitment and interaction of adaptor proteins in lipid rafts. Several TLRs exist as inactive, preformed heterodimers and homodimers in the absence of ligand (42, 79). TLR2 has been shown to exist as an inactive, loosely bound heterodimer with either TLR1 or TLR6 in the absence of ligand (34, 80). Upon lipopeptide binding, preformed TLR2/1 dimers undergo rearrangement subsequently bringing TLR2 and TLR1 in much closer proximity (42). This ligand-induced conformational rearrangement results in the immediate recruitment of signaling molecules including MyD88 and the cytoplasmic NOX2 organizer protein $p47^{phox}$ (56, 57, 60). Therefore, we next determined whether palmitic acid induces heterodimerization of TLR2 with TLR1 in close proximity that can be detected by TR-FRET. Because FRET occurs only when two receptors are within close proximity, it can be used as a direct readout for receptor dimerization. In addition, TR-FRET allows for detection of the dimerization process of native TLR1 and TLR2 in their biological context. Our results showed that palmitic acid or Pam_3CSK_4 increased TLR2/1-dependent TR-FRET within 10 min of incubation time, whereas DHA attenuates both palmitic acid– and Pam_3CSK_4-induced TR-FRET in THP-1 cells. To our knowledge, these results are the first to demonstrate that the dietary SFA palmitic acid directly induces, whereas DHA inhibits, the dimerization of TLR2 and TLR1. While our manuscript was in review, Yan et al. (81) reported that DHA inhibits inflammasome activation in murine BMDMs. These results together with our results demonstrate that DHA can inhibit both TLR-mediated pro–IL-1β expression (primary signal) and NALP3 inflammasome activation (secondary signal) in a cell-type–specific manner.

Dimerized TLR2 recruits MyD88 and $p47^{phox}$ into lipid raft fractions, which can be an additional readout of TLR activation. Concomitant recruitment of MyD88 and $p47^{phox}$ into lipid raft fractions (Fig. 6B, 6C) and corresponding increase in ROS generation by palmitic acid (Fig. 7A) strongly suggest that palmitic acid induces recruitment of downstream signaling molecules of TLRs including MyD88 and $p47^{phox}$. Recruitment of cytoplasmic $p47^{phox}$ to the plasma membrane allows for the association with $gp91^{phox}$ and $p22^{phox}$, and activation of the NOX2 enzyme complex (82). Recently, several studies have demonstrated recruitment and activation of NOX2 by agonist-induced activation of TLR4 and TLR2 (25, 51, 56, 57, 60, 83, 84). Our results showing that NOX2 inhibitor apocynin dose-dependently inhibits palmitic acid–induced dimerization of TLR2 (Fig. 7C) and IL-1β release (Fig. 7B) further support that palmitic acid–induced activation of TLR2 is also dependent on NOX2 activation. These results suggest that the downstream component of TLRs can exert positive feedback for activation of TLRs. Such a positive feedback mechanism can render rapid burst of ROS production upon microbial infection for killing invading pathogens. However, excessive production of ROS resulting from the activation of TLRs induced by endogenous molecules can trigger harmful effects leading to chronic sterile inflammation. How NOX-induced ROS can promote the dimerization of TLR4 or TLR2 is an intriguing question. One possibility is that NOX-induced ROS may promote the formation of disulfide bond between TLR2 and TLR1 as an analogy to many redox-sensitive proteins (85–88). Dimerization of TLR2 involves formation of a disulfide bond (89), which may be sensitively regulated by the immediate redox status of cell's microenvironment. Significant corollary to these results is that dietary components and physiological or metabolic processes that affect cellular redox status can directly modulate TLR-mediated inflammatory responses and their subsequent consequences.

We next determined whether endogenous SFAs derived from dietary fat recapitulate the effects of palmitic acid on dimerization of TLR2 with TLR1 in monocytes. The results showed that the lipolysis product of TGRL induced the dimerization of TLR2 with TLR1 as does palmitic acid (Fig. 8C). Recruitment of MyD88 by dimerized TLR2 led to activation of further downstream signaling molecules including NF-κB and MAPKs, and eventually the expression of target gene products (i.e., pro–IL-1β; Figs. 1A, 3D) and release of IL-1β in primary monocytes (Fig. 8B).

Collectively, our results reveal that SFAs derived from high-saturated fat meals can induce inflammasome-mediated IL-1β production in human monocytes by inducing the dimerization of TLR2 with TLR1. To our knowledge, this is the first report to demonstrate compelling evidence that palmitic acid can directly activate TLR2 by inducing receptor dimerization leading to the activation of downstream signaling pathways and target gene expression. These results further suggest that inflammasome-mediated IL-1β production in blood monocytes is dynamically modulated by the types of dietary fat we consume.

Disclosures

The authors have no financial conflicts of interest.

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