

# Palmitoleate Reverses High Fat-induced Proinflammatory Macrophage Polarization via AMP-activated Protein Kinase (AMPK)\*

Received for publication, February 20, 2015, and in revised form, May 15, 2015. Published, JBC Papers in Press, May 18, 2015, DOI 10.1074/jbc.M115.646992

Kenny L. Chan<sup>‡§1</sup>, Nicolas J. Pillon<sup>‡2</sup>, Darshan M. Sivaloganathan<sup>‡3</sup>, Sheila R. Costford<sup>‡</sup>, Zhi Liu<sup>‡</sup>, Marine Thérét<sup>¶4</sup>, Benedicte Chazaud<sup>¶</sup>, and Amira Klip<sup>‡§5</sup>

From the <sup>‡</sup>Cell Biology Program, The Hospital for Sick Children, Toronto, Ontario M5G 0A4, Canada, the <sup>§</sup>Department of Physiology, University of Toronto, Toronto, Ontario M5S 1A8, Canada, and the <sup>¶</sup>Centre de Génétique et de Physiologie Moléculaire et Cellulaire, Université Claude Bernard Lyon 1, Villeurbanne 69622, France

**Background:** Elevated saturated fats during obesity activate proinflammatory pathways in macrophages, contributing to insulin resistance.

**Results:** The monounsaturated fatty acid *cis*-palmitoleate antagonizes saturated fat-induced proinflammatory macrophage polarization through an AMPK-dependent mechanism.

**Conclusion:** Palmitoleate is a lipid mediator that confers an anti-inflammatory macrophage phenotype.

**Significance:** Understanding lipid-mediated macrophage polarization is critical to develop nutritional or cell-based strategies to combat insulin resistance.

A rise in tissue-embedded macrophages displaying “M1-like” proinflammatory polarization is a hallmark of metabolic inflammation during a high fat diet or obesity. Here we show that bone marrow-derived macrophages (BMDM) from high fat-fed mice retain a memory of their dietary environment *in vivo* (displaying the elevated proinflammatory genes *Cxcl1*, *Il6*, *Tnf*, *Nos2*) despite 7-day differentiation and proliferation *ex vivo*. Notably, 6-h incubation with palmitoleate (PO) reversed the proinflammatory gene expression and cytokine secretion seen in BMDM from high fat-fed mice. BMDM from low fat-fed mice exposed to palmitate (PA) for 18 h *ex vivo* also showed elevated expression of proinflammatory genes (*Cxcl1*, *Il6*, *Tnf*, *Nos2*, and *Il12b*) associated with M1 polarization. Conversely, PO treatment increased anti-inflammatory genes (*Mrc1*, *Tgfb1*, *Il10*, *Mgl2*) and oxidative metabolism, characteristic of M2 macrophages. Therefore, saturated and unsaturated fatty acids bring about opposite macrophage polarization states. Coincubation of BMDM with both fatty acids counteracted the PA-induced *Nos2* expression in a PO dose-dependent fashion. PO also prevented PA-induced I $\kappa$ B $\alpha$  degradation, RelA nuclear translocation, NO production, and cytokine secretion. Mechanistically, PO exerted its anti-inflammatory function through AMP-activated protein kinase as AMP kinase knockout or inhibition by Com-

pound C offset the PO-dependent prevention of PA-induced inflammation. These results demonstrate a nutritional memory of BMDM *ex vivo*, highlight the plasticity of BMDM polarization in response to saturated and unsaturated fatty acids, and identify the potential to reverse diet- and saturated fat-induced M1-like polarization by administering palmitoleate. These findings could have applicability to reverse obesity-linked inflammation in metabolically relevant tissues.

Insulin resistance is a major contributor to the development of type 2 diabetes, a multiorgan disease affecting more than 300 million people worldwide (1). Overnutrition and obesity lead to a systemic low-grade inflammation (2) characterized by elevated numbers of immune cells, predominantly macrophages, in metabolic tissues such as skeletal muscle, adipose, and liver (3–9). Tissue macrophage infiltration and proliferation have been associated with the appearance of peripheral insulin resistance (2, 4). Although both proinflammatory “M1-like” and anti-inflammatory “M2-like” macrophage counts rise in metabolically relevant tissues, there is a shift in their overall balance in favor of proinflammatory polarized macrophages (5, 6, 10). Conversely, the anti-inflammatory phenotype of muscle macrophages tracks with the insulin sensitivity index of the individual (11). To date, the role and plasticity of the M2-like macrophages within metabolic tissues during obesity remains poorly understood. Moreover, it is unknown whether the phenotype of resident and infiltrating macrophages is acquired within the metabolic tissues or whether high fat feeding also affects cells residing in the bone marrow.

*In vitro*, macrophages exposed to saturated fatty acids display increased proinflammatory gene expression and cytokine secretion (e.g. TNF $\alpha$ , IL6, and CXCL1/KC) (12–14). Conversely, polyunsaturated fatty acids suppress these inflammatory effects on monocytes/macrophages (15–20). A recent

\* This work was supported by Canadian Diabetes Association Grant OG-3-12-3838-AK and Canadian Institutes of Health Research Grant MOP-130493 (to A. K.), and by Fondation pour la Recherche Médicale Grant Equipe FRM DEQ20140329495 (to B. C.).

<sup>1</sup> Supported by a studentship from the Banting and Best Diabetes Centre (BBDC).

<sup>2</sup> Supported by a fellowship from the Research Training Centre from the Hospital for Sick Children.

<sup>3</sup> Supported by summer studentships from the BBDC and the SickKids Summer Research Program (SSuRe).

<sup>4</sup> Supported by a fellowship from the French Ministry of Research and Association Française contre les Myopathies.

<sup>5</sup> To whom correspondence should be addressed: Program in Cell Biology, The Hospital for Sick Children, Toronto, ON M5G 0A4, Canada. Tel.: 416-813-6392; E-mail: amira@sickkids.ca.

## Palmitoleate Reverses Inflammatory Macrophage Polarization

study ascribed this protection to activation of AMP-activated protein kinase (AMPK)<sup>6</sup> (21), and, indeed, AMPK activation *in vitro* and *in vivo* correlates with macrophage skewing toward an M2 phenotype (22–24).

A cluster of recent studies has revealed that, *in vivo*, adipose tissue secretes *cis*-palmitoleate (C16:1 n-7), a monounsaturated fatty acid that, in turn, protects against hepatic steatosis and improves whole-body insulin sensitivity (25–27). Moreover, the circulating levels of palmitoleate in humans strongly correlate with insulin action (28). Notably, however, the direct actions of palmitoleate on immune cells remain unknown.

Here we show that BMDM from high fat-fed mice exhibit increased markers of inflammation and, interestingly, that this phenotype is reversed by *ex vivo* incubation with palmitoleate. We next investigate the differential effects of palmitoleate alone and in combination with the saturated fatty acid palmitate (C16:0) on BMDM phenotypic skewing. The results indicate that palmitoleate confers an anti-inflammatory M2-like polarization to macrophages *ex vivo* that can counteract the inflammatory state brought upon by palmitate. Finally, we find that these anti-inflammatory effects are, in part, mediated by AMPK signaling.

### Experimental Procedures

**Animal Studies**—Rodent studies were approved by The Hospital for Sick Children Animal Care Committee. Male C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME, stock no. 000664) were fed a low fat (10% by kilocalories; catalog no. D12450B, Research Diets, New Brunswick, NJ) or high fat defined diet (60% by kilocalories; catalog no. D12492, Research Diets) for 18 weeks, starting from 9 weeks of age. Cages were maintained at 21–22 °C on a 12-h light cycle. Mice were euthanized via cervical dislocation following a 4-h fast.

**Isolation and Culture of BMDM**—Bone marrow was extracted from the femur and tibia of male chow-fed (catalog no. 5P07 Prolab RMH 1000, LabDiet, St. Louis, MO) 6- to 8-week-old C57BL/6 (Charles River Laboratories, St. Constant, QC, Canada) or C57BL/6J mice (The Jackson Laboratory) by centrifuging bones at 15,000 × *g* for 10 s. AMPKβ1 knockout (*Prkab1*<sup>-/-</sup>) mice (generated by Dr. Bruce E. Kemp, St. Vincent's Institute of Medical Research, Melbourne, Australia) and appropriate wild-type controls were made available by Dr. Gregory R. Steinberg (McMaster University, Hamilton, ON, Canada) and have been described previously (29). Extracted cells were cultured in RPMI medium (Wisent, Saint-Bruno, QC, Canada) supplemented with 10% heat-inactivated fetal bovine serum, 1× nonessential amino acids (Wisent), 1 mM sodium pyruvate (Wisent), 275 μM 2-mercaptoethanol (Life Technologies), and 1× antibiotic-antimycotic (Wisent). L929 fibroblasts were grown to confluence in low glucose DMEM (Wisent) supplemented with 10% fetal bovine serum and 1× antibiotic-antimycotic (Wisent) and then washed with PBS and serum-starved for 10 days. Medium was then collected and fil-

ter-sterilized, aliquoted, and stored at –20 °C as L929 conditioned medium. BMDM were seeded at 1 × 10<sup>6</sup> cells/ml and differentiated using 10% L929 conditioned medium for 7 days at 37 °C, 5% CO<sub>2</sub>.

**Reagents**—Palmitate (PA), *cis*-palmitoleate (PO), *cis*-oleate (catalog nos. P9767, P9417, and O1008, Sigma-Aldrich, St. Louis, MO), and palmitelaidic acid (*trans*-PO, catalog no. 9001798, Cayman Chemical, Ann Arbor, MI) stock solutions (200 mM) were prepared in 50% ethanol by heating at 50 °C. Fatty acid-free, low-endotoxin BSA (catalog no. A8806, Sigma-Aldrich) was dissolved in serum-free α minimum Eagle's medium (Wisent) to 10.5%. Fatty acid stocks were diluted 25× in the BSA solution and conjugated under agitation at 40 °C for 2 h. These solutions (lipid:BSA ratio, 5:1) were aliquoted and stored at –20 °C and then diluted further in cell culture medium. Compound C (catalog no. 171260, Millipore Canada, Etobicoke, ON, Canada) was dissolved in DMSO to a stock concentration of 10 mM before use in cell culture.

**Gene Expression Analysis by qPCR**—RNA was extracted using TRIzol (Life Technologies), and cDNA was synthesized by reverse transcription using the SuperScript VILO cDNA kit (Life Technologies) according to the instructions of the manufacturer. qPCR reactions were run using 10 ng of cDNA and predesigned TaqMan probes (Life Technologies) on a StepOne Plus real-time PCR system (Life Technologies) using the following parameters: one cycle of 95 °C for 20 s, followed by 40 cycles at 95 °C for 1 s and 60 °C for 20 s. Gene expression was normalized to that of the housekeeping genes *Abt1* and/or *Hprt*.

**Nitric Oxide Measurement**—Inducible nitric oxide synthase (iNOS) activity was assessed by the measurement of the NO breakdown product (NO<sub>2</sub><sup>-</sup>) into cell culture medium using the Griess reagent system. 1 mM sulfanilamide (catalog no. A13001, Alfa Aesar, Ward Hill, MA), 1 N HCl, and 7 mM naphthylethylenediamine (catalog no. 5230-16, Ricca Chemical Co., Arlington, TX) were added to cell culture samples before absorbance was read at 540 nm in a microplate reader.

**ELISA**—Following experimentation, cell culture supernatants were centrifuged at 15,000 × *g* for 10 min at 4 °C, aliquoted, and stored at –80 °C until analysis. Secreted IL6 and TNFα were analyzed using ELISA MAX Deluxe kits (BioLegend, San Diego, CA) according to the instructions of the manufacturer. Cell culture supernatants were analyzed for CXCL1/KC (keratinocyte chemoattractant) using Mouse KC ELISA (RayBiotech, Norcross, GA).

**Immunoblotting**—Cells were collected in lysis buffer (20 mM Tris, 138 mM NaCl, 2.7 mM KCl, 1 mM MgCl<sub>2</sub>, 5 mM EDTA, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 20 mM NaF, 1 mM dithiothreitol, 5% glycerol, 1% Nonidet P-40, and 1× protease inhibitor mixture (Sigma-Aldrich)) on ice. Lysates were centrifuged at 15,000 × *g* for 10 min, and protein concentration was determined in supernatants via bicinchoninic acid assay (Thermo Fisher Scientific, Wilmington, MA). 20 μg of protein was run on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Bio-Rad). Membranes were incubated with antibodies against IκBα (catalog no. 9242, Cell Signaling Technology, Danvers, MA), phospho-AMPKα (Thr-172, catalog no. 2531, Cell Signaling Technology), AMPKα (F6, catalog no. 2793, Cell Signaling

<sup>6</sup> The abbreviations used are: AMPK, AMP-activated protein kinase; BMDM, bone marrow-derived macrophage(s); PA, palmitate; PO, palmitoleate; qPCR, quantitative PCR; iNOS, inducible nitric oxide synthase; OCR, oxygen consumption rate; ANOVA, analysis of variance.

Technology) and actinin  $\alpha$  1 (loading control, catalog no. A5044, Sigma-Aldrich) overnight at 4 °C and then probed with peroxidase goat anti-rabbit IgG (catalog no. 111-035-003, Jackson ImmunoResearch Laboratories, West Grove, PA) or peroxidase goat anti-mouse IgG (catalog no. 115-035-003, Jackson ImmunoResearch Laboratories). Bands were visualized using an Odyssey Fc Imager (LI-COR, Lincoln, NE) and quantified using Odyssey Fc Image Studio version 4.0 (LI-COR).

**NF $\kappa$ B Immunofluorescence**—Cells grown on coverslips were fixed with 4% paraformaldehyde for 20 min at room temperature, quenched with 50 mM NH<sub>4</sub>Cl, blocked with 2% BSA in PBS for 20 min, and then incubated with anti-NF $\kappa$ B p65 antibody (catalog no. sc-372, Santa Cruz Biotechnology) for 1 h at room temperature, followed by three washes with 0.2% BSA in PBS. Cells were next incubated with goat anti-rabbit secondary antibody conjugated to Cy3 fluorophore (catalog no. 111-165-144, Jackson ImmunoResearch Laboratories) for 1 h in the dark at room temperature, followed by DAPI staining (Sigma-Aldrich) for 20 min in the dark at room temperature. Coverslips were subsequently washed with 0.2% BSA in PBS and mounted on slides using fluorescence mounting medium (Dako, Burlington, ON, Canada). Images were acquired using a Leica DMIRE2 inverted fluorescent microscope (Leica Microsystems, Wetzlar, Germany) equipped with Volocity 5.4.1 (PerkinElmer Life Sciences). Fields were selected and images quantified as described previously (30) using ImageJ 1.48 (National Institutes of Health, Bethesda, MD).

**Cell Metabolism Analysis**—Oxygen consumption rate (OCR) and extracellular acidification rate were measured using a Seahorse XF<sup>96</sup> analyzer (Seahorse Bioscience, Billerica, MA). Cells were seeded at 70,000 cells/well, incubated overnight in Seahorse 96-well cell culture plates, and then equilibrated for 2 h in XF base medium supplemented with 11 mM glucose and 1 mM sodium pyruvate. Following 20 min of baseline measurements, fatty acids were injected to a final concentration of 0.5 mM for 60 min.

**ATP Measurement**—Cells were lysed in 2% trichloroacetic acid on ice and then centrifuged at 15,000  $\times$  g for 10 min to remove cell debris. Lysates were neutralized with 1 M NaOH, and cytosolic ATP concentration was measured using the ENLITEN ATP assay system (Promega) according to the instructions of the manufacturer.

**Statistical Tests**—Data are expressed as mean  $\pm$  S.E. An unpaired Student's *t* test or one-way ANOVA with Bonferroni or Tukey post test was used to detect differences between means in datasets containing multiple groups. A two-way ANOVA with Bonferroni or Tukey post test was used to detect differences in datasets containing two variables. Statistical significance was set at *p* < 0.05. Graphs were prepared and data were analyzed using GraphPad Prism version 6 (GraphPad Software, La Jolla, CA).

## Results

**Macrophages from High Fat-fed Mice Display Inflammation, Which Can Be Reversed by Palmitoleate**—A hallmark feature of metabolic inflammation is a shift of tissue macrophages toward an M1-like polarization during a high fat diet or obesity. However, it is not known whether BMDM from high fat-fed mice

retain a memory of their dietary *in vivo* environment. This is an important question because the generation of BMDM from naïve bone marrow cells requires 7 days of incubation with growth factors *ex vivo*. To explore the possibility of this “dietary memory,” we generated BMDM from C57BL/6J mice fed either a high fat (60% kcal from fat) or low fat (10% kcal from fat) diet for 18 weeks. Strikingly, BMDM from high fat-fed mice displayed higher levels of proinflammatory gene expression (*Nos2*, *Il6*, *Cxcl1*, and *Tnf*) compared with macrophages from low fat-fed mice (Fig. 1).

Palmitoleate is a monounsaturated fatty acid produced by adipose tissue that has been correlated with the relief of insulin resistance in liver and skeletal muscle. We therefore explored whether palmitoleate could counteract the BMDM proinflammation caused by chronic high fat feeding. Notably, *ex vivo* incubation of BMDM with palmitoleate for 6 h reversed the proinflammatory gene expression (Fig. 1, A–D) and cytokine secretion (Fig. 1, B and D) seen in BMDM derived from high fat-fed mice. These findings highlight the plasticity of macrophage polarization and their capability to regulate their functional phenotype in response to lipid mediators such as palmitoleate.

**Saturated and Unsaturated Fatty Acids Differentially Regulate Macrophage Polarization**—On the basis of the observation that palmitoleate counteracted high fat-induced macrophage inflammation, we aimed to dissect the mechanisms underlying the effect of fats using isolated BMDM from regular chow-fed (6–8% kcal from fat) mice. We compared the action of palmitoleate *vis a vis* that of palmitate, a same chain length saturated fatty acid of known inflammatory effects. Palmitate is one the most abundant dietary saturated fatty acids; its plasma concentration approaches 0.4 mM during obesity, with total circulating free fatty acids reaching 1 mM (31, 32). In cell culture, a 0.5 mM palmitate concentration is widely used to recreate a high saturated fat environment (33–35). BMDM from mice were treated with BSA-conjugated PA, BSA-conjugated PO, or BSA alone as vehicle control, and macrophage polarization was assessed by expression of proinflammatory and anti-inflammatory genes through qPCR. BMDM exposed to 0.5 mM PA for 18 h showed elevated expression of proinflammatory genes (*Cxcl1*, *Il6*, *Tnf*, *Nos2*, *Il12b*, and *Ciita*) associated with M1 macrophage polarization relative to the PO-treated BMDM (Fig. 2). Conversely, anti-inflammatory M2 genes (*Mrc1*, *Tgfb1*, *Il10*, *Arg1*, *Chi3l3*, and *Mgl2*) rose with PO treatment relative to the PA-treated group. These results demonstrate that saturated and unsaturated fatty acids lead to distinct macrophage polarization states, with PA producing an M1-like phenotype and PO producing an M2-like phenotype.

**Palmitoleate Selectively Prevents Palmitate-induced Macrophage Inflammation**—Given that PO both increased anti-inflammatory markers in macrophages and reversed the proinflammatory signature seen in macrophages generated from high fat-fed mice, we next tested whether it could offset the proinflammatory effects brought about by PA. In BMDM coin-cubated with both fatty acids, PO reduced the PA-induced increases in the classical M1 marker *Nos2*/iNOS in a dose-dependent fashion at both 6 and 18 h of coin-cubation, and this response was completely ablated by 0.5 mM PO (Fig. 3A). Inter-



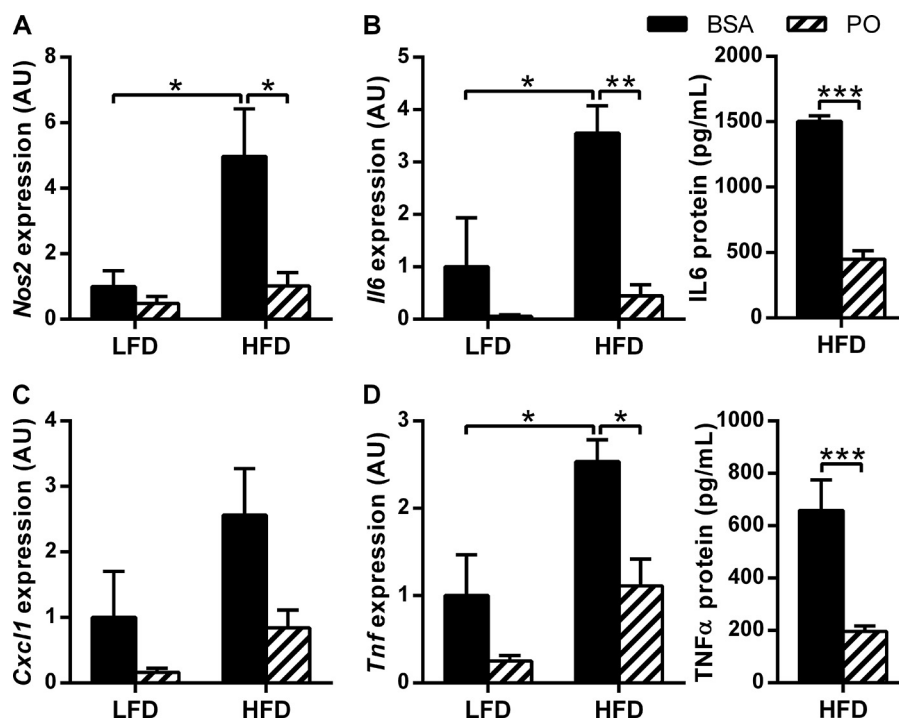


FIGURE 1. **Palmitoleate reverses high fat diet-induced macrophage inflammation.** BMDM were isolated and cultured from C57BL/6J mice fed either a low fat diet (LFD, 10% kcal from fat) or high fat diet (HFD, 60% kcal from fat) for 18 weeks. BMDM were treated with BSA or 0.5 mM PO for 6 h. iNOS (A), IL6 (B), CXCL1 (C), and TNF $\alpha$  (D) expression and/or secretion were determined by qPCR or ELISA, respectively. Results are expressed as mean  $\pm$  S.E.  $n = 3$ ; two-way ANOVA; Bonferroni post test; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ , unpaired Student's  $t$  test; \*\*\*,  $p < 0.001$ .

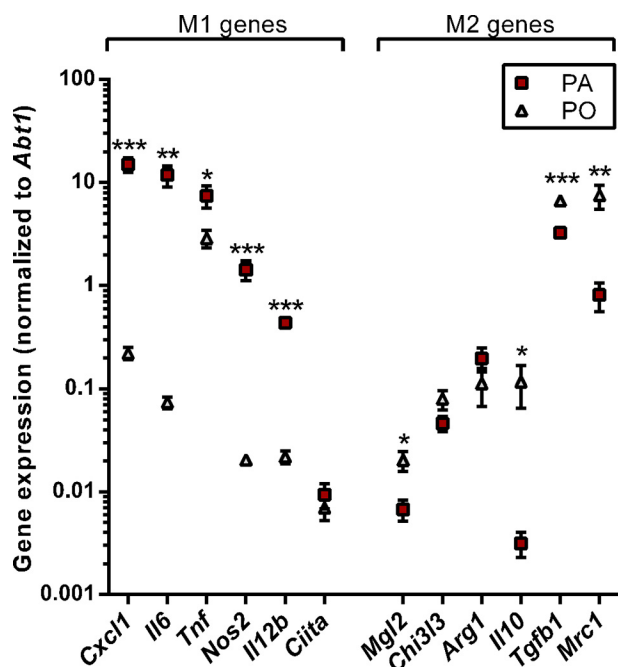


FIGURE 2. **Palmitate and palmitoleate differentially regulate macrophage polarization.** BMDM were cultured and treated with 0.5 mM PA or 0.5 mM PO for 18 h. M1 (*Cxcl1*, *Il6*, *Tnf*, *Nos2*, *Il12b*, and *Ciita*) and M2 (*Mgl2*, *Chl3l3*, *Arg1*, *Il10*, *Tgfb1*, and *Mrc1*)-associated gene expression was analyzed by qPCR and normalized to the housekeeping gene *Abt1*. Results are expressed as mean  $\pm$  S.E.  $n = 7-10$ ; unpaired Student's  $t$  test; \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

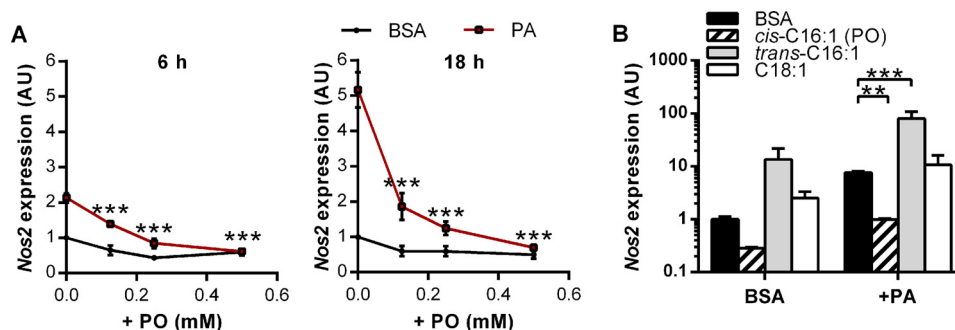
estingly, this anti-inflammatory effect was specific to the *cis* form of palmitoleate because neither *trans*-palmitoleate nor the monounsaturated fatty acid oleate reduced PA-induced *Nos2* expression (Fig. 3B). Therefore, the anti-inflammatory

effects of PO on macrophages appear to be a selective property not shared by other monounsaturated fatty acids, including the abundant dietary fatty acid oleate.

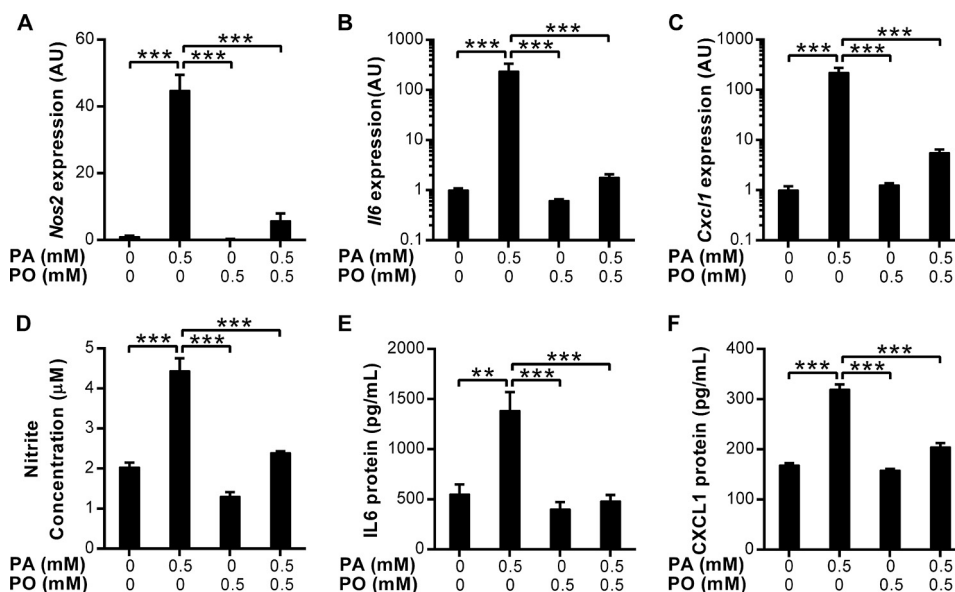
The preventive action of PO was further characterized by examining its effects on the genes most up-regulated by PA, seen in Fig. 2. In addition to *Nos2*, PO (0.5 mM) prevented PA-induced expression of *Il6* and *Cxcl1* (Fig. 4, A–C). If functional, the increase in *Nos2*/iNOS should lead to NO production, a key product of the immune response. We therefore analyzed nitrite levels as a reflection of NO production as well as the release of the proinflammatory cytokines IL6 and CXCL1/KC. The results paralleled those in gene expression, with PO preventing PA-induced NO production and cytokine secretion (Fig. 4, D–F).

Each of the proinflammatory genes analyzed above are under transcriptional regulation by NF $\kappa$ B. Therefore, we examined whether PO would inhibit activation of the NF $\kappa$ B pathway in BMDM. The results clearly revealed that PA induced I $\kappa$ B $\alpha$  degradation and nuclear translocation of the NF $\kappa$ B p65 (RelA) subunit. Addition of PO during the incubation with PA prevented both I $\kappa$ B $\alpha$  degradation and RelA nuclear translocation (Fig. 5), reversing the values to those observed in the BSA-treated control group. Collectively, these results demonstrate that PO exerts a protective, anti-inflammatory effect on macrophages by antagonizing saturated fat-induced NF $\kappa$ B activation, iNOS production of NO, and pro inflammatory cytokine gene expression as well as the consequent cytokine secretion.

**Palmitoleate Increases Fatty Acid Oxidation**—A hallmark of M2 macrophages is a shift from glycolytic toward oxidative metabolism (36, 37). Because PO induced an M2-like macrophage polarization, we analyzed whether PO increased oxi-



**FIGURE 3. Cis-palmitoleate dose-dependently decreases palmitate-induced *Nos2*/iNOS expression.** A, BMDM were given increasing doses of PO in the presence or absence of 0.5 mM PA in equivalent amounts of BSA for 6 or 18 h.  $n = 3$ ; two-way ANOVA; Tukey post test; \*\*\*,  $p < 0.001$  versus PA + 0.0 mM PO (A). B, BMDM were treated with 0.5 mM *cis*-palmitoleate (C16:1), *trans*-palmitoleate (C16:1), or oleate (C18:1) in the presence or absence of 0.5 mM PA. *Nos2* expression was measured by qPCR. Results are expressed as mean  $\pm$  S.E. in arbitrary units (AU) relative to BSA + 0.0 mM PO at the corresponding time point. Two-way ANOVA; Tukey post test; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

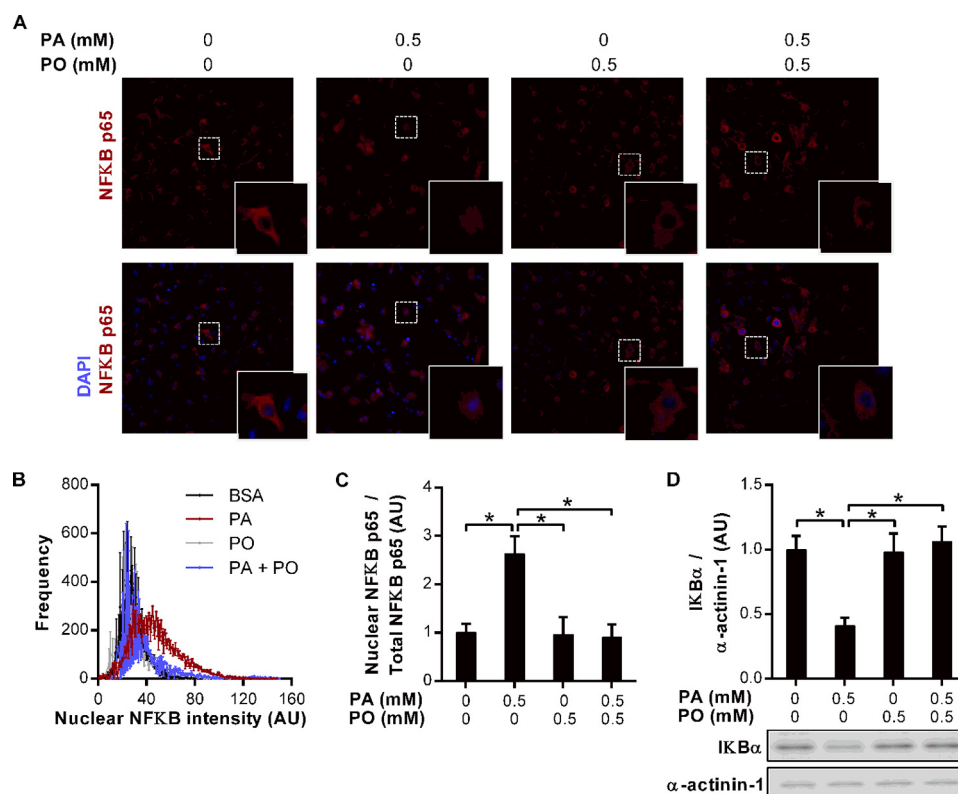


**FIGURE 4. Palmitoleate prevents palmitate-induced proinflammatory gene expression, nitric oxide production, and cytokine secretion.** BMDM were treated with BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO in equivalent amounts of BSA for 18 h. A–C, expression of *Nos2* (A), *Il6* (B), and *Cxcl1* (C) was determined by qPCR and shown relative to BSA in arbitrary units (AU). D, NO production was assessed by measuring nitrite concentration in cell culture supernatants by Griess assay. E and F, secreted IL6 (E) and CXCL1 (F) proteins were measured by ELISA. Results are expressed as mean  $\pm$  S.E.  $n = 4$ ; one-way ANOVA; Bonferroni post test; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ .

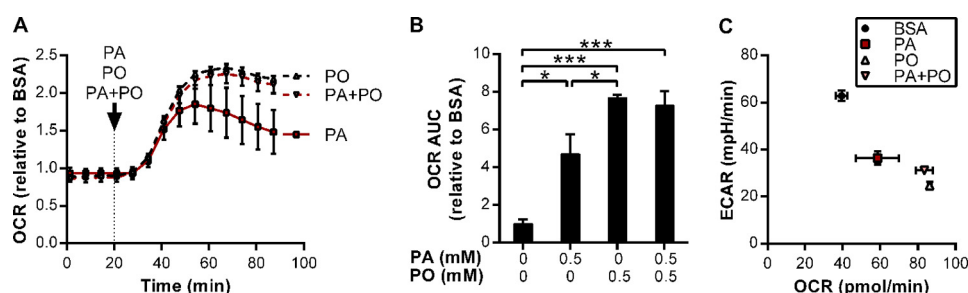
dative respiration in macrophages. As expected, the lipids increased the OCR relative to the BSA control in both the PA and PO treatment groups. However, the OCR was greater in macrophages given PO compared with those given PA (Fig. 6, A and B). Interestingly, when coincubated with both PA and PO together, the OCR resembled the phenotype seen in the PO-treated group (Fig. 6C) despite the presence of twice as much lipid as under either the PA or PO conditions. These results indicate that, when exposed to PO, macrophages increase fatty acid oxidation and rely less on glycolysis.

**AMPK Mediates the Anti-inflammatory Effects of Palmitoleate**—Because direct AMPK activation up-regulates fatty acid oxidation in tissues (38, 39) and can antagonize saturated fatty acid-induced inflammation (23, 24), we tested whether PO would exert its anti-inflammatory function on BMDM through AMPK. After 30 min, PO elevated AMPK $\alpha$  phosphorylation in BMDM relative to the BSA-treated control group, whereas PA decreased AMPK $\alpha$  phosphorylation. Under coincubation with both PA and PO, AMPK $\alpha$  phosphorylation levels were similar

to those seen with PO alone (Fig. 7A), highlighting that AMPK activation is specific to the unsaturated fatty acid. By 18 h, there were no significant differences in AMPK $\alpha$  phosphorylation between treatment groups (data not shown). Although a drop in cellular ATP levels is a signal for AMPK activation, we could not find any differences in cytosolic ATP levels between any of the treatment groups. The nucleotide levels did not change at 30 min of incubation with palmitoleate, when AMPK $\alpha$  phosphorylation is clearly observed (Fig. 7B), nor at earlier time points (data not shown), suggesting that AMPK may be activated by PO through an ATP-independent mechanism. To evaluate whether AMPK activity was necessary for the anti-inflammatory effects of PO, we used an AMPK inhibitor, Compound C, in conjunction with fatty acid treatments of BMDM. As shown above, PO prevented the I $\kappa$ B $\alpha$  degradation induced by PA. However, AMPK inhibition diminished the ability of PO to prevent PA-induced I $\kappa$ B $\alpha$  degradation (Fig. 7C). Accordingly, the PO-induced prevention of PA-induced *Nos2* expression (Fig. 7D) and NO production (Fig.



**FIGURE 5. Palmitoleate prevents palmitate-induced NF $\kappa$ B activity.** BMDM were treated with BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO (PA + PO) in equivalent amounts of BSA for 18 h. **A**, BMDM grown on coverslips were fixed, permeabilized, and stained with anti-NF $\kappa$ B p65 antibody and DAPI before images were captured on a Leica DMIRE2 fluorescence microscope using a  $\times 40$  oil objective. **B** and **C**, NF $\kappa$ B nuclear translocation was measured by p65 staining intensity in the nuclear region of interest defined by DAPI staining (**B**) and expressed as a ratio of total cellular NF $\kappa$ B p65 (**C**). **D**, cell lysates were analyzed for I $\kappa$ B $\alpha$  degradation by immunoblotting. Results are expressed as mean  $\pm$  S.E. in arbitrary units (AU) relative to BSA.  $n = 3$ ; one-way ANOVA; Bonferroni post test; \*,  $p < 0.05$ .



**FIGURE 6. Palmitoleate increases oxidative metabolism in macrophages.** OCR as a measure of oxidative metabolism and extracellular acidification rate (ECAR) as a measure of glycolytic metabolism of BMDM were assessed using a Seahorse XF<sup>96</sup> extracellular flux analyzer. **A** and **B**, basal OCR was measured for 20 min, followed by 60 min in the presence of BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO (PA + PO) (**A**), and the area under the curve (AUC) was calculated (**B**). **C**, OCR versus extracellular acidification rate is shown at 60 min following BSA, PA, PO, or PA + PO addition. Results are expressed as mean  $\pm$  S.E.  $n = 3$ ; one-way ANOVA; Tukey post test; \*,  $p < 0.05$ ; \*\*\*,  $p < 0.001$ .

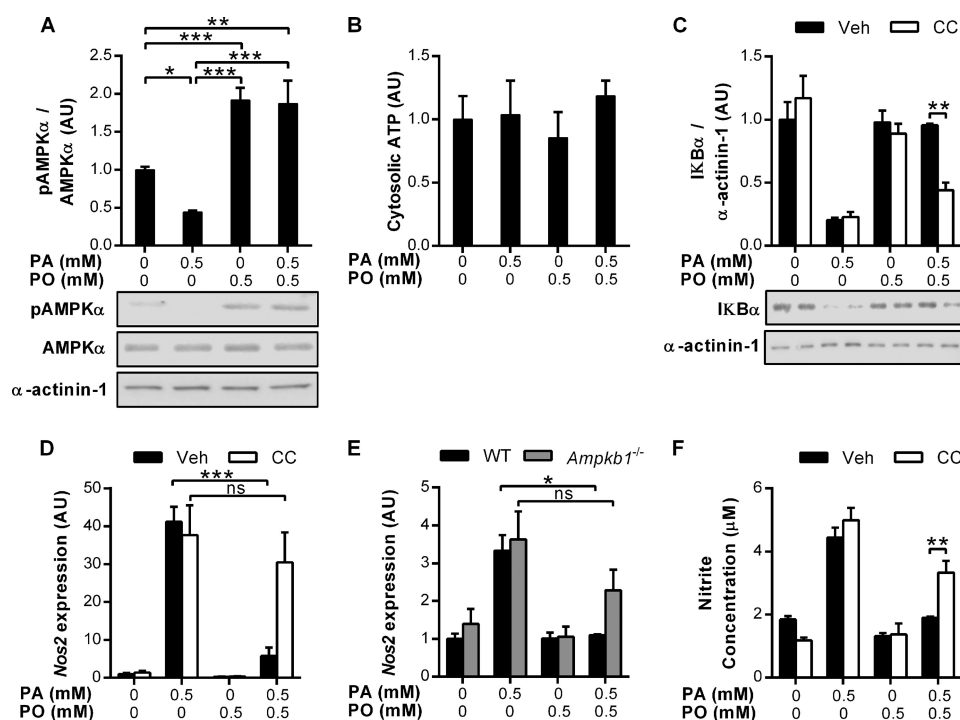
7F) were voided when AMPK was inhibited using Compound C. Furthermore, a similar trend is seen in AMPK $\beta$ 1 knockout macrophages (Fig. 7E), which lack the  $\beta$ 1 subunit essential for assembling the AMPK heterotrimer, resulting in AMPK $\alpha$  degradation (29). These results demonstrate that AMPK partially mediates the anti-inflammatory effects of PO on BMDM.

## Discussion

The main observations of this study are that the omega-7 monounsaturated fatty acid *cis*-palmitoleate can readily antagonize or reverse proinflammatory macrophage polarization induced by a high fat diet *in vivo* or by palmitate *in vitro* and that

AMPK participates in this reversal of macrophage metabolic inflammation.

**Inflammatory Status of BMDM from High Fat-fed Mice**—Over the past decade, high fat diets have been linked to tissue macrophage inflammation contributing to peripheral insulin resistance (2, 4). *Ex vivo*, saturated fatty acids evoke a proinflammatory response in primary and cell line macrophages (12, 13), and products thereof confer insulin resistance to muscle cells in culture (14, 40). The inflammatory status of macrophages induced by a high fat diet has been shown previously in tissue-resident macrophages, including muscle and adipose tissue macrophages and liver Kupffer cells (5, 6, 8). Strikingly, we observed *ex vivo* persistence of a dietary



**FIGURE 7. AMPK mediates the anti-inflammatory effects of palmitoleate.** A and B, BMDM were treated with BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO for 30 min, AMPKα phosphorylation was determined by immunoblotting (A), and cytosolic ATP concentration was measured (B). BMDM were pretreated with 5 μM compound C (CC) or DMSO vehicle (Veh) for 30 min and then incubated with BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO in equivalent amounts of BSA for 18 h. C, IκBα degradation was assessed by immunoblotting. D and F, Nos2/iNOS expression and NO production were measured by qPCR and Griess assay, respectively. E, WT or *Ampkb*<sup>1-/-</sup> macrophages were treated with BSA alone, 0.5 mM PA, 0.5 mM PO, or 0.5 mM PA + 0.5 mM PO, and Nos2/iNOS expression was evaluated by qPCR. Results are expressed as mean ± S.E. in arbitrary units (AU) relative to control (BSA, Veh-BSA, or WT-BSA). n = 3–6; one-way ANOVA; Bonferroni post test; \*, p < 0.05; \*\*, p < 0.01; \*\*\*, p < 0.001 (A). Two-way ANOVA; Bonferroni post test; \*\*, p < 0.01; \*\*\*, p < 0.001 (B). ns, not significant.

memory displayed by macrophages derived from the bone marrow of high fat-fed mice, which remained following 7 days of cellular differentiation *ex vivo*.

Current thinking has been that immune cells become polarized upon exposure to fatty acids in the circulation or target tissues. Our findings raise the possibility that these fatty acids may first affect precursor cells in the bone marrow, where the majority of circulating monocytes originate, potentially initiating inflammatory programs in monocytes before they reach the circulation. Future studies should investigate how fats act on the bone marrow and how the *ex vivo* dietary memory is maintained, whether through shifts in bone marrow cell populations, epigenetic modifications, or other mechanisms.

Using BMDM from high fat-fed mice as a model of metabolically inflamed macrophages, our next aim was to identify novel anti-inflammatory lipid mediators. Cao *et al.* (25) have highlighted *cis*-palmitoleate as an adipose-derived “lipokine” with beneficial insulin-sensitizing effects on a whole-body level (25). Because limiting macrophage inflammation has been adopted as a strategy to combat insulin resistance in mouse models *in vivo*, we tested whether palmitoleate would have anti-inflammatory effects in BMDM from high fat-fed mice. Remarkably, a 6-h incubation with palmitoleate completely reversed the pro-inflammatory polarization conferred to bone marrow cells through 18 weeks of high fat feeding. Because of the persistence of the *in vivo* high fat feeding over 7 days of culture *ex vivo* and the rapid reversal with palmitoleate treatment, it is likely that the pathways activated during diet-induced BMDM inflamma-

tion differ from those inhibited by palmitoleate. Potentially, signals elicited by palmitoleate may counteract the diet-activated pathways.

**Mechanism of Palmitoleate Counteraction of the Proinflammatory Action of Palmitate on BMDM**—To investigate the mechanism of action of palmitoleate to counteract fat-induced inflammation, we tested its effect on palmitate-challenged BMDM from chow-fed mice. Palmitate-challenged BMDM displayed activation of the NFκB pathway, leading to elevated proinflammatory gene expression, triggering NO production and cytokine secretion. This behavior is in line with results observed in macrophage cell lines and BMDM treated with diverse saturated fatty acids (41, 42). Notably, we found that coincubation with palmitoleate inhibited each of these palmitate-induced proinflammatory effects. It is conceivable that this action of palmitoleate may arise from its antagonizing action on IκBα degradation activity because this is an upstream signal for inflammatory cytokine production (43, 44). Palmitoleate can similarly antagonize the palmitate-induced NFκB activation in myotubes (55).

Toll-like receptor 4 (TLR4) has been linked to saturated fat-induced inflammation in many cell types, and its inhibition or depletion protects against diet-induced inflammation and insulin resistance (12, 45, 46). However, it is unlikely that the counteraction of palmitate effects by palmitoleate is simply the result of TLR4 inhibition because palmitoleate did not completely abolish BMDM inflammation evoked by the TLR4 agonist lipopolysaccharide (data not shown). Similarly, we deem it



unlikely that palmitoleate simply prevents or competes for palmitate uptake into macrophages because the disappearance of fatty acids from cell culture medium was additive upon incubation with both fatty acids (data not shown). Further, palmitoleate exerted anti-inflammatory effects independently of the presence of palmitate, as in the case of BMDM from chow-fed and high fat-fed mice.

**Role of AMPK in Palmitoleate Anti-inflammatory Action—**Instead, our results indicate that the anti-inflammatory effects of palmitoleate involve AMPK signaling because palmitoleate administration activated AMPK, and an AMPK inhibitor offset the anti-inflammatory input of palmitoleate on palmitate-challenged BMDM. The involvement of AMPK in the anti-inflammatory and counterinflammatory actions of palmitoleate is reminiscent of the reported AMPK activation by polyunsaturated fats (21) and the association of AMPK with anti-inflammatory M2 macrophage polarization, oxidative metabolism, and NF $\kappa$ B inhibition (22, 47, 48). The mechanism through which any lipid, including palmitoleate, activates AMPK is still unknown. AMPK functions as a master cellular energy sensor, becoming activated during low-energy states, and, therefore, appears as a converging signal linking nutrient sensing to inflammation in immune cells.

Although a drop in the ATP/AMP ratio is a signal for AMPK activation and some studies show that polyunsaturated fats inhibit glycolysis or can deplete cellular energy through mitochondrial uncoupling (49–51), we did not detect any changes in cytosolic ATP levels under any of the treatments studied. Moreover, palmitoleate increased oxidative metabolism in BMDM, as expected from AMPK activation. We hypothesize that AMPK activation by palmitoleate occurs through a nucleotide-independent mechanism. Indeed, other signaling pathways impact AMPK activity status, and a number of upstream kinases, including Ca<sup>2+</sup>-calmodulin dependent kinase kinase  $\beta$ , can activate AMPK (52, 53).

It is also conceivable that palmitoleate acts through a receptor to activate signals upstream of AMPK. Oh *et al.* (54) identified the G protein-coupled receptor 120 (GPR120) as a sensor mediating anti-inflammatory effects of polyunsaturated fats (54). Although that study focused on omega-3 polyunsaturated fatty acids, palmitoleate appeared during their screen of fatty acids that can activate GPR120 in HEK293 cells, raising the possibility that GPR120 may act as the receptor for monounsaturated fatty acids as well. Curiously, the monounsaturated fatty acid oleate also appeared to activate GPR120 in that study. However, we did not observe any anti-inflammatory effects of oleate on BMDM, indicating that GPR120 activation may not necessarily lead to an anti-inflammatory phenotype under all circumstances. We also found that other monounsaturated fats, including *trans*-palmitoleate, are unable to antagonize palmitate-induced *Nos2* expression, highlighting the specificity of *cis*-palmitoleate to offset palmitate-induced inflammation of BMDM. This result correlates with a recent observation that palmitoleate, but not oleate, can attenuate diet-induced glucose and insulin intolerance in mice (27).

Finally, considering the association between glycolytic metabolism with proinflammatory M1 macrophage polarization and of oxidative metabolism with anti-inflammatory M2

polarization (37, 39), future experiments should address whether changes in these metabolic pathways are a cause or consequence of the phenotypic skewing of cells.

Overall, our study demonstrates an AMPK-mediated anti-inflammatory effect of the monounsaturated fatty acid *cis*-palmitoleate in macrophages that can offset the macrophage inflammation conferred by palmitate or a prior high fat diet *in vivo*. These findings may provide the basis for dietary interventions or for cell-specific strategies to treat inflammatory diseases, including obesity-linked type 2 diabetes.

**Author Contributions—**K. L. C. participated in the design of the study, coordinated and carried out experiments, performed data analysis, and wrote the manuscript. N. J. P. participated in scientific discussion and assisted with the experiments shown in Fig. 1. D. M. S. helped with the experiments and data analysis shown in Figs. 2–4. S. R. C. participated in the study design and assisted with the experiments shown in Fig. 1. Z. L. provided technical assistance throughout the study and participated in the experiments shown in Figs. 2–4. M. T. and B. C. advised on the experiments for Fig. 7 and provided input in the writing of the manuscript. A. K. conceived and coordinated the study and participated in the writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

**Acknowledgments—**We thank Dr. Philip J. Bilan for insightful discussions throughout the course of this study, Dr. Yi Sun for help with immunofluorescence, and the SPARC Biocenter at The Hospital for Sick Children for help with performing the cell metabolism analysis. We also thank Dr. Bruce Kemp and Dr. Gregory Steinberg for generating and making available AMPK $\beta$ 1<sup>−/−</sup> mice.

## References

1. Danaei, G., Finucane, M. M., Lu, Y., Singh, G. M., Cowan, M. J., Paciorek, C. J., Lin, J. K., Farzadfar, F., Khang, Y.-H., Stevens, G. A., Rao, M., Ali, M. K., Riley, L. M., Robinson, C. A., Ezzati, M., and Global Burden of Metabolic Risk Factors of Chronic Diseases Collaborating Group (Blood Glucose) (2011) National, regional, and global trends in fasting plasma glucose and diabetes prevalence since 1980: systematic analysis of health examination surveys and epidemiological studies with 370 country-years and 2.7 million participants. *Lancet* **378**, 31–40
2. Xu, H., Barnes, G. T., Yang, Q., Tan, G., Yang, D., Chou, C. J., Sole, J., Nichols, A., Ross, J. S., Tartaglia, L. A., and Chen, H. (2003) Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J. Clin. Invest.* **112**, 1821–1830
3. Weisberg, S. P., McCann, D., Desai, M., Rosenbaum, M., Leibel, R. L., and Ferrante, A. W., Jr. (2003) Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **112**, 1796–1808
4. Kanda, H., Tateya, S., Tamori, Y., Kotani, K., Hiasa, K., Kitazawa, R., Kitazawa, S., Miyachi, H., Maeda, S., Egashira, K., and Kasuga, M. (2006) MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* **116**, 1494–1505
5. Lumeng, C. N., Bodzin, J. L., and Saltiel, A. R. (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **117**, 175–184
6. Fink, L. N., Costford, S. R., Lee, Y. S., Jensen, T. E., Bilan, P. J., Oberbach, A., Blüher, M., Olefsky, J. M., Sams, A., and Klip, A. (2014) Pro-inflammatory macrophages increase in skeletal muscle of high fat-fed mice and correlate with metabolic risk markers in humans. *Obesity* **22**, 747–757
7. Oh, D. Y., Morinaga, H., Talukdar, S., Bae, E. J., and Olefsky, J. M. (2012) Increased macrophage migration into adipose tissue in obese mice. *Diabetes* **61**, 346–354
8. Stanton, M. C., Chen, S.-C., Jackson, J. V., Rojas-Triana, A., Kinsley, D.,



- Cui, L., Fine, J. S., Greenfeder, S., Bober, L. A., and Jenh, C.-H. (2011) Inflammatory Signals shift from adipose to liver during high fat feeding and influence the development of steatohepatitis in mice. *J. Inflamm. (Lond)* **8**, 8
9. Morinaga, H., Mayoral, R., Heinrichsdorff, J., Osborn, O., Franck, N., Hah, N., Walenta, E., Bandyopadhyay, G., Pessentheiner, A. R., Chi, T. J., Chung, H., Bogner-Strauss, J. G., Evans, R. M., Olefsky, J. M., and Oh, D. Y. (2015) Characterization of distinct subpopulations of hepatic macrophages in HFD/obese mice. *Diabetes* **64**, 1120–1130
10. Fujisaka, S., Usui, I., Bukhari, A., Ikutani, M., Oya, T., Kanatani, Y., Tsuneyama, K., Nagai, Y., Takatsu, K., Urakaze, M., Kobayashi, M., and Tobe, K. (2009) Regulatory mechanisms for adipose tissue M1 and M2 macrophages in diet-induced obese mice. *Diabetes* **58**, 2574–2582
11. Fink, L. N., Oberbach, A., Costford, S. R., Chan, K. L., Sams, A., Blüher, M., and Klip, A. (2013) Expression of anti-inflammatory macrophage genes within skeletal muscle correlates with insulin sensitivity in human obesity and type 2 diabetes. *Diabetologia* **56**, 1623–1628
12. Shi, H., Kokeva, M. V., Inouye, K., Tzamelis, I., Yin, H., and Flier, J. S. (2006) TLR4 links innate immunity and fatty acid-induced insulin resistance. *J. Clin. Invest.* **116**, 3015–3025
13. Wen, H., Gris, D., Lei, Y., Jha, S., Zhang, L., Huang, M. T., Brickey, W. J., and Ting, J. P. (2011) Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling. *Nat. Immunol.* **12**, 408–415
14. Kewalramani, G., Fink, L. N., Asadi, F., and Klip, A. (2011) Palmitate-activated macrophages confer insulin resistance to muscle cells by a mechanism involving protein kinase C  $\theta$  and  $\epsilon$ . *PLoS ONE* **6**, e26947
15. Choi, E.-Y., Jin, J.-Y., Choi, J.-I., Choi, I. S., and Kim, S.-J. (2014) DHA suppresses *Prevotella intermedia* lipopolysaccharide-induced production of proinflammatory mediators in murine macrophages. *Br. J. Nutr.* **111**, 1221–1230
16. Nakakuki, M., Kawano, H., Notsu, T., and Imada, K. (2013) Eicosapentaenoic acid suppresses palmitate-induced cytokine production by modulating long-chain acyl-CoA synthetase 1 expression in human THP-1 macrophages. *Atherosclerosis* **227**, 289–296
17. Snodgrass, R. G., Huang, S., Choi, I.-W., Rutledge, J. C., and Hwang, D. H. (2013) Inflammasome-mediated secretion of IL-1 $\beta$  in human monocytes through TLR2 activation; modulation by dietary fatty acids. *J. Immunol.* **191**, 4337–4347
18. Solanki, P., Aminoshariae, A., Jin, G., Montagnese, T. A., and Mickel, A. (2013) The effect of docosahexaenoic acid (DHA) on expression of IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$  in normal and lipopolysaccharide (LPS)-stimulated macrophages. *Quintessence Int.* **44**, 393
19. Weldon, S. M., Mullen, A. C., Loscher, C. E., Hurley, L. A., and Roche, H. M. (2007) Docosahexaenoic acid induces an anti-inflammatory profile in lipopolysaccharide-stimulated human THP-1 macrophages more effectively than eicosapentaenoic acid. *J. Nutr. Biochem.* **18**, 250–258
20. Mullen, A., Loscher, C. E., and Roche, H. M. (2010) Anti-inflammatory effects of EPA and DHA are dependent upon time and dose-response elements associated with LPS stimulation in THP-1-derived macrophages. *J. Nutr. Biochem.* **21**, 444–450
21. Xue, B., Yang, Z., Wang, X., and Shi, H. (2012) Omega-3 polyunsaturated fatty acids antagonize macrophage inflammation via activation of AMPK/SIRT1 pathway. *PLoS ONE* **7**, e45990
22. Mounier, R., Théret, M., Arnold, L., Cuvellier, S., Bultot, L., Göransson, O., Sanz, N., Ferry, A., Sakamoto, K., Foretz, M., Viollet, B., and Chazaud, B. (2013) AMPK $\alpha$ 1 regulates macrophage skewing at the time of resolution of inflammation during skeletal muscle regeneration. *Cell Metab.* **18**, 251–264
23. Dong, J., Zhang, X., Zhang, L., Bian, H.-X., Xu, N., Bao, B., and Liu, J. (2014) Quercetin reduces obesity-associated ATM infiltration and inflammation in mice: a mechanism including AMPK $\alpha$ 1/SIRT1. *J. Lipid Res.* **55**, 363–374
24. Yang, Z., Wang, X., He, Y., Qi, L., Yu, L., Xue, B., and Shi, H. (2012) The full capacity of AICAR to reduce obesity-induced inflammation and insulin resistance requires myeloid SIRT1. *PLoS ONE* **7**, e49935
25. Cao, H., Gerhold, K., Mayers, J. R., Wiest, M. M., Watkins, S. M., and Hotamisligil, G. S. (2008) Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell* **134**, 933–944
26. Yang, Z.-H., Miyahara, H., and Hatanaka, A. (2011) Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay mice with genetic type 2 diabetes. *Lipids Health Dis.* **10**, 120
27. Souza, C. O., Teixeira, A. A., Lima, E. A., Batatinha, H. A., Gomes, L. M., Carvalho-Silva, M., Mota, I. T., Streck, E. L., Hirabara, S. M., and Rosa Neto, J. C. (2014) Palmitoleic acid (n-7) attenuates the immunometabolic disturbances caused by a high-fat diet independently of PPAR $\alpha$ . *Mediators Inflamm.* **2014**, 582197
28. Stefan, N., Kantartzis, K., Celebi, N., Staiger, H., Machann, J., Schick, F., Cegan, A., Elcnerova, M., Schleicher, E., Fritsche, A., and Häring, H.-U. (2010) Circulating palmitoleate strongly and independently predicts insulin sensitivity in humans. *Diabetes Care* **33**, 405–407
29. Dzakmo, N., van Denderen, B. J., Hevener, A. L., Jørgensen, S. B., Honeyman, J., Galic, S., Chen, Z.-P., Watt, M. J., Campbell, D. J., Steinberg, G. R., and Kemp, B. E. (2010) AMPK  $\beta$ 1 deletion reduces appetite, preventing obesity and hepatic insulin resistance. *J. Biol. Chem.* **285**, 115–122
30. Noursadeghi, M., Tsang, J., Hausteiner, T., Miller, R. F., Chain, B. M., and Katz, D. R. (2008) Quantitative imaging assay for NF- $\kappa$ B nuclear translocation in primary human macrophages. *J. Immunol. Methods* **329**, 194–200
31. Ubhayasekera, S. J., Staaf, J., Forslund, A., Bergsten, P., and Bergquist, J. (2013) Free fatty acid determination in plasma by GC-MS after conversion to Weinreb amides. *Anal. Bioanal. Chem.* **405**, 1929–1935
32. Kruszynska, Y. T., Olefsky, J. M., and Frias, J. P. (2003) Effect of obesity on susceptibility to fatty acid-induced peripheral tissue insulin resistance. *Metabolism* **52**, 233–238
33. Erbay, E., Babaev, V. R., Mayers, J. R., Makowski, L., Charles, K. N., Snitow, M. E., Fazio, S., Wiest, M. M., Watkins, S. M., Linton, M. F., and Hotamisligil, G. S. (2009) Reducing endoplasmic reticulum stress through a macrophage lipid chaperone alleviates atherosclerosis. *Nat. Med.* **15**, 1383–1391
34. Galic, S., Fullerton, M. D., Schertzer, J. D., Sikkema, S., Marcinko, K., Walkley, C. R., Izon, D., Honeyman, J., Chen, Z.-P., van Denderen, B. J., Kemp, B. E., and Steinberg, G. R. (2011) Hematopoietic AMPK  $\beta$ 1 reduces mouse adipose tissue macrophage inflammation and insulin resistance in obesity. *J. Clin. Invest.* **121**, 4903–4915
35. Koves, T. R., Ussher, J. R., Noland, R. C., Slentz, D., Mosedale, M., Ilkayeva, O., Bain, J., Stevens, R., Dyck, J. R., Newgard, C. B., Lopaschuk, G. D., and Muoio, D. M. (2008) Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. *Cell Metab.* **7**, 45–56
36. Huang, S. C., Everts, B., Ivanova, Y., O'Sullivan, D., Nascimento, M., Smith, A. M., Beatty, W., Love-Gregory, L., Lam, W. Y., O'Neill, C. M., Yan, C., Du, H., Abumrad, N. A., Urban, J. F., Jr., Artyomov, M. N., Pearce, E. L., and Pearce, E. J. (2014) Cell-intrinsic lysosomal lipolysis is essential for alternative activation of macrophages. *Nat. Immunol.* **15**, 846–855
37. Galván-Peña, S., and O'Neill, L. A. (2014) Metabolic reprogramming in macrophage polarization. *Front. Immunol.* **5**, 420
38. Steinberg, G. R., and Schertzer, J. D. (2014) AMPK promotes macrophage fatty acid oxidative metabolism to mitigate inflammation: implications for diabetes and cardiovascular disease. *Immunol. Cell Biol.* **92**, 340–345
39. O'Neill, L. A., and Hardie, D. G. (2013) Metabolism of inflammation limited by AMPK and pseudo-starvation. *Nature* **493**, 346–355
40. Talbot, N. A., Wheeler-Jones, C. P., and Cleasby, M. E. (2014) Palmitoleic acid prevents palmitic acid-induced macrophage activation and consequent p38 MAPK-mediated skeletal muscle insulin resistance. *Mol. Cell. Endocrinol.* **393**, 129–142
41. Suganami, T., Nishida, J., and Ogawa, Y. (2005) A paracrine loop between adipocytes and macrophages aggravates inflammatory changes: role of free fatty acids and tumor necrosis factor  $\alpha$ . *Arterioscler. Thromb. Vasc. Biol.* **25**, 2062–2068
42. Huang, S., Rutkowski, J. M., Snodgrass, R. G., Ono-Moore, K. D., Schneider, D. A., Newman, J. W., Adams, S. H., and Hwang, D. H. (2012) Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways. *J. Lipid Res.* **53**, 2002–2013
43. Baldwin, A. S. (1996) The NF- $\kappa$ B and I  $\kappa$ B proteins: new discoveries and insights. *Annu. Rev. Immunol.* **14**, 649–683
44. Xie, Q. W., Kashiwabara, Y., and Nathan, C. (1994) Role of transcription

- factor NF- $\kappa$ B/Rel in induction of nitric oxide synthase. *J. Biol. Chem.* **269**, 4705–4708
45. Tsukumo, D. M., Carvalho-Filho, M. A., Carvalheira, J. B., Prada, P. O., Hirabara, S. M., Schenka, A. A., Araújo, E. P., Vassallo, J., Curi, R., Velloso, L. A., and Saad, M. J. (2007) Loss-of-function mutation in Toll-like receptor 4 prevents diet-induced obesity and insulin resistance. *Diabetes* **56**, 1986–1998
46. Pal, D., Dasgupta, S., Kundu, R., Maitra, S., Das, G., Mukhopadhyay, S., Ray, S., Majumdar, S. S., and Bhattacharya, S. (2012) Fetuin-A acts as an endogenous ligand of TLR4 to promote lipid-induced insulin resistance. *Nat. Med.* **18**, 1279–1285
47. Sag, D., Carling, D., Stout, R. D., and Suttles, J. (2008) Adenosine 5'-monophosphate-activated protein kinase promotes macrophage polarization to an anti-inflammatory functional phenotype. *J. Immunol.* **181**, 8633–8641
48. Salminen, A., Hyttinen, J. M., and Kaarniranta, K. (2011) AMP-activated protein kinase inhibits NF- $\kappa$ B signaling and inflammation: impact on healthspan and lifespan. *J. Mol. Med.* **89**, 667–676
49. Dentin, R., Benhamed, F., Pégrier, J.-P., Foullet, F., Viollet, B., Vaulont, S., Girard, J., and Postic, C. (2005) Polyunsaturated fatty acids suppress glycolytic and lipogenic genes through the inhibition of ChREBP nuclear protein translocation. *J. Clin. Invest.* **115**, 2843–2854
50. Andrade-Vieira, R., Han, J. H., and Marignani, P. A. (2013) Omega-3 polyunsaturated fatty acid promotes the inhibition of glycolytic enzymes and mTOR signaling by regulating the tumor suppressor LKB1. *Cancer Biol. Ther.* **14**, 1050–1058
51. Piquet, M. A., Fontaine, E., Sibille, B., Filippi, C., Keriell, C., and Leverve, X. M. (1996) Uncoupling effect of polyunsaturated fatty acid deficiency in isolated rat hepatocytes: effect on glycerol metabolism. *Biochem. J.* **317**, 667–674
52. Witters, L. A., Kemp, B. E., and Means, A. R. (2006) Chutes and ladders: the search for protein kinases that act on AMPK. *Trends Biochem. Sci.* **31**, 13–16
53. Towler, M. C., and Hardie, D. G. (2007) AMP-activated protein kinase in metabolic control and insulin signaling. *Circ. Res.* **100**, 328–341
54. Oh, D. Y., Talukdar, S., Bae, E. J., Imamura, T., Morinaga, H., Fan, W., Li, P., Lu, W. J., Watkins, S. M., and Olefsky, J. M. (2010) GPR120 is an omega-3 fatty acid receptor mediating potent anti-inflammatory and insulin-sensitizing effects. *Cell* **142**, 687–698
55. Macrae, K., Stretton, C., Lipina, C., Blachnio-Zabielska, A., Baranowski, M., Gorski, J., Marley, A., and Hundal, H. S. (2013) Defining the role of DAG, mitochondrial function, and lipid deposition in palmitate-induced proinflammatory signaling and its counter-modulation by palmitoleate. *J. Lipid Res.* **54**, 2366–2378