



Palmitoleic acid reduces the inflammation in LPS stimulated macrophages by inhibition of NFκB, independently of PPARs.

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Palmitoleic acid reduces the inflammation in LPS stimulated macrophages by inhibition of NFκB, independently of PPARs.

Running title: Anti-inflammatory effects of palmitoleate

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ABSTRACT

Palmitoleic acid (PM, 16:1n-7) has anti-inflammatory properties that could be linked to higher expression of PPAR α , an inhibitor of NF κ B. Macrophages play a major role in the pathogenesis of chronic inflammation, however, the effects of PM on macrophages are underexplored. Thus, we aimed to investigate the effects of PM in activated macrophages as well the role of PPAR α . Primary macrophages were isolated from C57BL6 (WT) and PPAR α knockout (KO) mice, cultured under standard conditions and exposed to LPS (2.5 μ g/mL) and PM 600 μ M conjugated with albumin for 24 hours. The stimulation with LPS increased the production of IL-6 and IL-1 β while PM decreased the production of IL-6 in WT macrophages. In KO macrophages, LPS increased the production of TNF- α and IL-6 and PM decreased the production of TNF α . The expression of inflammatory markers such NF κ B and IL1 β were increased by LPS and decreased by PM in both WT and KO macrophages. PM reduced the expression of MyD88 and caspase-1 in KO macrophages, and the expression of TLR4 and HIF-1 α in both WT and KO macrophages, although LPS had not effect. CD86, an inflammatory macrophage marker, was reduced by PM independently of genotype. PM increased PPAR γ and reduced PPAR β gene expression in macrophages of both genotypes, and increased ACOX-1 expression in KO macrophages. In conclusion, PM promotes anti-inflammatory effects in macrophages exposed to LPS through inhibition of inflammasome pathway, which was independent of PPAR α , PPAR γ and AMPK, thus the molecular mechanisms of anti-inflammatory response caused by PM is still unclear.

Keywords: monounsaturated fatty acid; palmitoleate; immune cell; macrophage; PPAR α knockout mice; inflammasome complex.

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1. INTRODUCTION

Inflammation is a common feature of many chronic diseases. It has been described as a hallmark of obesity, insulin resistance, atherosclerosis and cancer (1). It is well known that saturated fatty acids, which are prevalent in the western diet and found in abundance in industrialized food, promote toxic effects and increase the inflammatory response in several tissues and cell types (2-4). In fact, palmitic acid is described as an activator of the inflammasome pathway, increasing the expression of interleukin (IL) 1 β via toll like receptor-4 (TLR4) activation (2, 4). The inflammasome is a large multi-protein complex activated by both exogenous and endogenous danger signals cleaving caspase-1 and, therefore, promoting the maturation of pro-inflammatory cytokines such IL-1 β , IL-18 and IL-33 (5).

Besides the modulation of the inflammasome complex, the successful resolution of inflammatory processes requires the inhibition of pro-inflammatory signaling by M2 macrophages, which have high capacity to counteract the functions of classical pro-inflammatory M1 macrophages. The omega-3 polyunsaturated fatty acids seem to promote this phenotypic switch in macrophages by inhibiting both NF-kB and inflammasome activation (1). Similarly, palmitoleic acid (PM), an omega-7 monounsaturated fatty acid (16:1n-7) described as a lipokine that improves whole-body insulin sensitivity (6), has been shown to decrease the levels of several pro-inflammatory cytokines (IL-1 β , TNF- α and IL-6) (6-8), as well as the expression of TLR4 (8) in liver of obese mice.

Besides this anti-inflammatory effect in the liver, PM also reversed the pro-inflammatory gene expression and cytokine secretion in cultured macrophages (9) , as well as those extracted from bone marrow of high fat-fed mice (10). Despite description of those beneficial properties, the direct effects of PM on M1 macrophages are underexplored and the mechanisms involved not fully understood. Some authors have described the metabolic effects of PM as mediated by activation of peroxisome

proliferator-activated receptor- α (PPAR α) (11), a transcription factor that besides its pro-oxidative properties also modulates inflammation by blocking NF κ B (12).

Thus, we aimed to investigate the effects of PM in activated macrophages and the role of PPAR α . Here we showed that palmitoleic acid decreased the M1/M2 ratio and reduced the production of pro-inflammatory cytokines by lowering the expression of NF κ B and genes from the inflammasome complex. Those anti-inflammatory effects of palmitoleic acid are not dependent on PPAR α or PPAR γ and should be further explored.

2. RESULTS

Preliminary experiments established that 600 μ M was the higher concentration of PM that could be used without a toxic effect (suppl. 1).

LPS increased expression of PPAR α in cultured macrophages from WT mice (Figure 1). Expression of PPAR α was further increased if PM was present (Figure 1).

Incubation with LPS for 24 hours increased the production of TNF- α and IL1 β ($p < 0.001$) by macrophages from PPAR α knockout (KO) mice, and the production of IL-6 ($p < 0.01$) by macrophages from both genotypes (Figure 2). PM (600 μ M) blunted the production of IL-6 ($p < 0.05$) in WT and KO macrophages stimulated with LPS, as well the production of TNF- α ($p < 0.001$) and IL1 β ($p < 0.001$) in KO macrophages stimulated with LPS (Figure 2).

Lower concentrations of PM also had some anti-inflammatory effects (suppl. 2). The production of IL-1 β in WT macrophages stimulated with LPS was decreased by 200 μ M PM ($p < 0.001$) (suppl. 2C). In KO macrophages stimulated with LPS, the production of TNF- α was decreased ($p < 0.001$) by PM at 400 μ M (Suppl. 2B), while the production of IL1 β was decreased at both 200 and 400 μ M PM ($p < 0.001$, $p < 0.01$) (suppl. 2C).

Besides its well known pro-inflammatory effects, LPS stimulation increased the production of IL-10 in macrophages of both genotypes ($p<0.001$ in WT and $p<0.05$ in KO) (suppl 3), while the incubation with several concentrations of PM did not show any effect (suppl. 3A). The anti-inflammatory cytokine IL-1Ra was not stimulated by LPS, however, incubation of LPS plus PM (600 μ M) decreased ($p<0.05$) IL-1Ra production in KO, while no other concentration of PM affected the production of IL-1Ra (suppl. 3B).

Since PM at 600 μ M had significant effects on the production of pro-inflammatory cytokines induced by LPS in macrophages of both genotypes (Figure 2), we used this concentration to analyse the expression of several genes related to the inflammatory response (Figure 3). Even co-incubated with LPS, PM decreased the expression of TLR4 ($p<0.001$) and HIF1 α ($p<0.05$) in macrophages from mice of both genotypes when compared to the control (Figure 3A and 3D, respectively). PM+LPS also decreased expression of MyD88 ($p<0.01$) and Caspase-1 ($p<0.05$) unstimulated (control) and LPS stimulated macrophages from KO mice (Figure 3B and 3C, respectively). LPS increased the expression of NF κ B ($p<0.05$) (Figure 3E) in WT macrophages. Likewise the expression of IL-1 β was increased by LPS ($p<0.001$) (Figure 3F) in macrophages from KO mice. PM decreased NF κ B and IL-1 β ($p<0.05$ and $p<0.001$) in both genotypes when compared to LPS alone.

The expression of MyD88 ($p<0.05$), caspase-1($p<0.01$) and HIF1 α ($p<0.01$) were around 2-fold higher in macrophages of KO mice when compared to those from WT mice (Figure 3). However, after the incubation with LPS and PM for 24 hours, no difference in the expression of MyD88, caspase-1 and HIF1 α was observed between the genotypes (Figure 3).

The decreased production of pro-inflammatory cytokines (Figure 2) and the lower expression of NF κ B and some other genes related to inflammation (Figure 3) caused by the PM could be due to or result in modulation the macrophage phenotype. Therefore, we investigated the expression of classical markers used to diferenciate M1 macrophages (CD86) (Figure 4A) from M2 macrophages (arginase) (Figure 4B).

Although the expression of both genes (CD86 and arginase) was reduced ($p < 0.05$) by LPS in macrophages from both WT and KO mice, the expression of the M1 marker CD86 was decreased further ($p < 0.05$) by PM in macrophages from both genotypes stimulated with LPS (Figure 4A). Likewise, the ratio between the expression of CD86/arginase (M1/M2) was higher ($p < 0.001$) in WT macrophages stimulated with LPS and it was lowered ($p < 0.05$) by PM (Figure 4C). Macrophages from PPAR α KO mice had a higher ($p < 0.05$) expression of Acyl CoA oxidase-1 (ACOX-1) (Figure 4D) and AMP activated Kinase (AMPK) (Figure 4E), the exposure to LPS decreased ($p < 0.05$) the expression ACOX-1, while PM restored ($p < 0.05$) the expression of this gene (Figure 4D). On other hand, the expression of AMPK was decreased ($p < 0.01$) by PM in KO mice macrophages (Figure 4E).

Although PM enhanced the expression of PPAR α , it was able to induce anti-inflammatory effects even in PPAR α KO mice. Interestingly when we investigated the expression of PPAR γ , PPAR β and genes regulated by those, opposite effects were observed (Figure 5). In both genotypes, the expression of PPAR γ was increased ($p < 0.001$) by LPS + PM (Figure 5A), while PPAR β expression was decreased ($p < 0.05$) (Figure 5B). The upregulation of PPAR γ and downregulation of PPAR β promoted by PM was clear in PPAR α Knockout mice, since LPS did not promoted effect on the expression of those genes, differently of macrophages from WT mice, in which the LPS promoted effects similar to PM, increasing PPAR γ ($p < 0.001$) expression and downregulating PPAR β ($p < 0.01$) (Figure 5).

The upregulation of PPAR γ promoted by palmitoleic acid in PPAR α Knockout macrophages indicated a possible mechanism for the anti-inflammatory effects of PM independently of PPAR α . However, in macrophages extracted from PPAR α Knockout mice, palmitoleic acid still was able to reduce the production of IL-6 ($p < 0.001$), IL1 β ($p < 0.001$) and TNF α ($p < 0.05$) stimulated by LPS, even when co-incubated with GW9662, a PPAR γ inhibitor (Figure 6A, 6B and 6C). As unexpected, this anti-inflammatory effect of palmitoleic acid independently of PPAR γ , was further confirmed

in macrophages extracted from PPAR γ Lys Cre⁺ (Knockout of PPAR γ specific for macrophages) and PPAR γ Lys Cre⁻ (wild type), in with palmitoleic acid reduced the production of IL-6 (p<0.01), IL1 β (p<0.05), TNF α (p<0.001) and MCP-1 (p<0.01) in both genotypes (Figure 6D, 6E, 6F, 6G). By last, we verified that besides indepently of PPAR γ , the anti-inflammatory effects of palmitoleic acid also occurs in absence of AMPK, as this monounsaturated fatty acid still reduced the production of IL-6 (p<0.01), IL1 β (p<0.01), TNF α (p<0.001) and MCP-1 (p<0.01) stimulated by LPS in PPAR γ Cre⁺ Knockout macrophages, even when co-incubated with an AMPK inhibitor, the compound C (Figure 6H, 6I, 6J, 6K).

3. DISCUSSION

Since its first description as a lipokine, PM has been reported to improve insulin sensitivity (6) and protect from atherosclerosis and cardiovascular disease (13). Here, we identify another beneficial effect of PM, which decreased inflammatory responses of in macrophages. This occurred on macrophages isolated both from WT and PPAR α KO mice, indicating that although the expression of the anti-inflammatory transcription factor PPAR α is increased by PM, the effects of this monounsaturated ω 7 fatty acid seem to be independent of PPAR α .

Beneficial effects of PM were previously observed in cultures of J774 macrophages, where it counteracted the M1 polarization induced by palmitate, increasing the insulin response in C2C12 myoblasts (9). Similar effects were observed in endothelial cells, where PM partly prevented the pro-apoptotic effects of palmitic acid, reducing endothelial dysfunction (14). Although the mechanisms by which PM mediated these effects are unknown, in cultured 3T3L1 adipocytes, PM increased lipolysis and the expression of lipases via activation of PPAR α (11).

PPAR α , a transcription factor that is constitutive expressed in all tissues, is anti-inflammatory exerting this effect by binding to the response element of NF κ B,

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3 blocking the transcription of inflammatory genes (15). In the current study, PM lowered
4 the production of pro-inflammatory cytokines, and reduced the expression of genes
5 from the inflammasome complex and NF κ B, even in PPAR α KO mice, what indicate
6 the activation of PPAR α expression is not the only mechanism responsible for the anti-
7 inflammatory effect of PM (Figure 7).
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13 In accordance with the results for macrophages seen in the current study,
14 previous work by our group showed that PM decreased liver inflammation in obese
15 mice independently of an effect on PPAR α (8). In the liver, PM decreased TLR4 gene
16 expression in both WT and PPAR α KO mice and reduced the protein expression of
17 TLR4 and NF κ B (p65) in PPAR α KO mice (8). Likewise, in the present study, PM
18 inhibited production of several pro-inflammatory cytokines, as well as down regulation
19 of TLR4, MyD88, caspase-1 and HIF1 α in macrophages from both WT and PPAR α KO
20 mice, probably due to downregulation of NF κ B.
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29 Omega-3 polyunsaturated fatty acids which have well known anti-inflammatory
30 effects, had effects similar to PM, reducing the expression of genes from the
31 inflammasome pathway by inhibiting the activation of NF κ B in bone marrow derived
32 macrophages (BMDM) (1). The anti-inflammatory effect promoted by omega-3 PUFAs
33 seems to be mediated by upregulation of PPAR- γ (16-18), nuclear receptor that
34 similarly to PPAR α , interferes with the NF κ B activation (1, 18), reducing the production
35 of pro-inflammatory cytokines (TNF α and IL6) (17-19). In accordance, we observed PM
36 increased the expression of PPAR γ , especially in macrophages from animals that lacks
37 PPAR α , in which the expression of NF κ B and IL1 β were also blunted by this fatty acid.
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47 Similarly to Omega 3 PUFAS (17-19), it was observed that PM protected
48 BMDM against the pro-inflammatory effects of saturated fatty acids by inducing
49 differentiation to the anti-inflammatory M2 phenotype (10). Our findings suggest the
50 same effect of palmitoleic acid in peritoneal macrophages, since it lowered the ratio
51 between the expression of CD86 and arginase, indicatives of the M1/M2 ratio.
52 Inflammation driven by M1 macrophages is counterbalanced by alternative polarization
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to M2 macrophages, which promote resolution of inflammation. Beneficial properties of M2 macrophages are reported in several inflammatory disorders and deregulation in the M1/M2 phenotypic balance is described as a mechanism for pathogenesis of chronic inflammatory diseases (20). It is important to mention, that besides the classical M1/M2 genes here analyzed, we also observed that PM modulated genes related to metabolic pathways that are nowadays considers characteristics of M1 (glycolytic metabolism) or M2 (oxidative metabolism) phenotypes (21). In fact PM downregulated HIF-1 α , gene related to glycolysis (M1), while upregulated ACOX-1 in KO macrophages, gene of the oxidative metabolism (M2) (21, 22).

Those effects on the polarization of macrophages can be also related to PPAR γ , which acts in an anti-inflammatory manner regulating the macrophages differentiation to M2 phenotype (23). In fact, PPAR γ , an isoform of PPARs highly expressed in macrophages, has an important role in macrophage differentiation (24). It was observed previously that the activation of PPAR γ in cultured macrophages, by thiazolidinediones or 15-deoxy-prostaglandin J₂, resulted in reduced production of several pro-inflammatory cytokines (25) and down regulation of pro-inflammatory gene expression by inhibition of transcription factors, AP-1, STAT and NF κ B (26). The current study showed that PM stimulated the expression of PPAR γ , in macrophages from WT and PPAR α KO mice. On other hand, the opposite effect was observed for PPAR β , another PPAR that is expressed in several tissues and was reduced by PM in macrophages from mice of both genotypes.

These observations indicate that the modulation of PPAR γ expression by PM might have an important anti-inflammatory effect in PPAR α KO mice. In fact, the expression of ACOX-1 was increased in macrophages from PPAR α KO mice by PM, although this oxidative enzyme expression is well known as directly activated by PPAR α (27). Similar effects have been already described in the liver of PPAR α knockout mice treated with thiazolidinediones (PPAR γ activators) (28), which indicates some degree of cross-activation between PPAR γ and PPAR α (28, 29), and suggests

the residual higher PPAR γ expression induced by PM in macrophages of PPAR α KO mice may be sufficient to partially restore PPAR α function (Figure 7).

Although the importance of PPAR α and PPAR γ might have on the inflammation and macrophages polarization, our findings indicate palmitoleic acid might have an effect that is independent of both PPARs, since the palmitoleic acid reduced the inflammation stimulated by LPS in PPAR α KO macrophages even when co-incubated with GW9662 (inhibitor of PPAR γ) and palmitoleic acid also reduced the inflammation stimulated by LPS in PPAR γ Lys Cre⁺ Knockout mice.

Besides PPARs, several studies have linked the metabolic effects of palmitoleic acid with AMPK, an energy status sensor increased by palmitoleic acid in liver, adipose tissue and skeletal muscle, which seems to induce anti-inflammatory effects (30-32). In vitro experiments demonstrated that LPS promoted downregulation of AMPK expression in leukocytes (33). AMPK inhibits the inflammatory response induced by LPS in neutrophils (34), and in macrophages the activation of AMPK seems to promote polarization to an anti-inflammatory M2 phenotype.

AMPK downstream targets, such as SIRT-1 and FOXO, are described to inhibit the NF κ B signaling (35). Our group previously observed palmitoleic acid supplementation was able to reduce liver inflammation (8) while increased the AMPK phosphorylation (31), in accordance, it has been described that PM phosphorylates and activates AMPK in adipose tissue (30) and in BMDM (10), but in the current study, the expression of AMPK was increased in macrophages from PPAR α KO mice and was decreased by PM. This could indicate a regulatory response to over activation of AMPK, reducing gene expression as a feedback mechanism, however, the AMPK inhibitor, Compound C, did not impair the anti-inflammatory effect of palmitoleic acid in PPAR γ cre⁺ macrophages, indicating the effects of this fatty acid in vitro are not dependent of those factors and might occur by a unknown mechanism that modulates the inflammasome complex genes.

The anti-inflammatory effects of palmitoleic acid were independently of the genotype, indicating that palmitoleic acid is able to reduce the inflammation induced by LPS independently of PPAR α or PPAR γ . The inhibition of inflammation by palmitoleic acid are also not mediated through activation of AMPK, thus, is possible that the effects of palmitoleic acid are caused by modulation of genes from the inflammasome complex by an unknown mechanism, or by increase the oxidative phosphorylation, blocking the activation of HIF1 α and the production of pro-inflammatory cytokines such TNF α and IL1 β (Figure 7).

In conclusion, the results of the present work showed palmitoleic acid decreased the production of pro-inflammatory cytokines, lowered the expression of NF κ B and other genes related to inflammation, reduced expression of genes from the inflammasome complex and decreased the M1/M2 phenotypic expression in macrophages stimulated with LPS by mechanism that modulates but are not dependent of PPAR α or PPAR γ .

4. METHODS

4.1. Animals

All experimental protocols were approved by the Animal Care Committee of the Institute of Biomedical Sciences, University of São Paulo, Brazil (049/05/03/CEUA). The mice were male 10-wk-old mice maintained at 22 \pm 2°C in a 12h light cycle, fed with a balanced chow pellet diet (Nuvilab CR1, Nuvital, Colombo, PR, Brazil) with *ad libitum* water supply. The following breeds were utilized: C57BL/6 wild-type (WT) (Animal Facility of the Institute of Biomedical Sciences, University of São Paulo, São Paulo, Brazil), PPAR α knockout (KO) mice (The Jackson Laboratory, Bar Harbor, ME, stock no. 008154), and mice with a selective deletion of PPAR γ in monocytes and mature macrophages (PPAR γ Cre+). To produce the PPAR γ Cre+ mice, the PPAR γ Lox/P

mice (The Jackson Laboratory, Bar Harbor, ME, USA, stock no. 004584) were crossed with LysMcre mice (The Jackson Laboratory, Bar Harbor, ME, USA stock no. 004781) and were selected by genotyping using primers and protocols indicated by the manufacturer.

4.2. Isolation and culture of intraperitoneal macrophages

Mice were fasted for 6 hours, anesthetized by isoflurane inhalation and euthanized before macrophage isolation. Macrophages were isolated from the intraperitoneal cavity of the mice with RPMI medium. The cells were counted in a hemocytometer using trypan blue (0.4%) exclusion and $5 \times 10^5/\text{cm}^2$ viable macrophages were cultured in medium (RPMI) supplement with fetal bovine serum (10%), and antibiotics solutions (1%) in standard conditions (37°C in humidified 95% air/5% CO₂). For the experiments, macrophages were exposed to PBS (control), LPS (2.5 µg/mL, E. coli O111:B4) with or without PM (600 µM) conjugated to BSA (bovine serum albumin). Inhibitors of PPAR γ (GW9662) and AMPK (compound C) (Sigma Aldrich, Saint Louis, MO, USA) were also utilized. After 24 hours, medium and macrophages were collected for further analysis.

4.3. Enzyme-Linked Immunosorbent Assay (ELISA)

The concentrations of IL-1 β , TNF- α , IL-6, MCP-1 IL-1Ra and IL-10 in cell culture supernatants were measured by ELISA (DuoSet ELISA®, R&D Systems, Minneapolis, MN, USA) performed according to the manufacturer's instructions.

4.4. RNA isolation, reverse transcription, and real-time PCR

The expression of genes related to metabolic and inflammatory pathways was assessed by qRT-PCR with SYBR Green marker. For this, total RNA was extracted from the cells as described by Chomczynski and Sacchi (1987) (36), quantified in a spectrophotometer (260 nm), and cDNA was synthesized from the total RNA using

reverse transcriptase. The sequences of the primers are shown in Table 1; gene expression was quantified by the comparative method using the expression of GAPDH as the standard (37).

4.5. Statistical analysis

Normal distribution and variance homogeneity were tested and the appropriate statistical test (one-way ANOVA or two-way ANOVA) was employed followed by post-hoc testing. Data are mean ± SEM (standard error) and analyses were performed using GraphPad Prism 5.0. Differences were considered significant when p<0.05.

5. ACKNOWLEDGEMENTS

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6. REFERENCE

1. Williams-Bey Y, Boularan C, Vural A, et al. Omega-3 free fatty acids suppress macrophage inflammasome activation by inhibiting NF-kappaB activation and enhancing autophagy. *PLoS One*. 2014; **9**:e97957. doi: 10.1371/journal.pone.0097957. eCollection 2014.

2. Ricchi M, Odoardi MR, Carulli L, et al. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. *J Gastroenterol Hepatol*. 2009; **24**:830-40. doi: 10.1111/j.1440-746.2008.05733.x. Epub 2009 Jan 13.

3. Wen H, Gris D, Lei Y, et al. Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling
Palmitate-activated macrophages confer insulin resistance to muscle cells by a mechanism involving protein kinase C theta and epsilon. *Nat Immunol*. 2011; **12**:408-15. doi: 10.1038/ni.2022. Epub 11 Apr 10.

4. Kewalramani G, Fink LN, Asadi F, Klip A. Palmitate-activated macrophages confer insulin resistance to muscle cells by a mechanism involving protein kinase C theta and epsilon. *PLoS One*. 2011; **6**:e26947. doi: 10.1371/journal.pone.0026947. Epub 2011 Oct 26.

5. Yu HB, Finlay BB. The caspase-1 inflammasome: a pilot of innate immune responses. *Cell Host Microbe*. 2008; **4**:198-208. doi: 10.1016/j.chom.2008.08.007.

6. Cao H, Gerhold K, Mayers JR, Wiest MM, Watkins SM, Hotamisligil GS. Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism. *Cell*. 2008; **134**:933-44. doi: 10.1016/j.cell.2008.07.048.

7. Guo X, Li H, Xu H, et al. Palmitoleate induces hepatic steatosis but suppresses liver inflammatory response in mice. *PLoS One*. 2012; **7**:e39286. doi: 10.1371/journal.pone.0039286. Epub 2012 Jun 29.

8. Souza CO, Teixeira AA, Lima EA, et al. Palmitoleic acid (n-7) attenuates the immunometabolic disturbances caused by a high-fat diet independently of PPARalpha. *Mediators Inflamm*. 2014; **2014**:582197. doi: 10.1155/2014/582197. Epub 2014 Jul 24.
9. Talbot NA, Wheeler-Jones CP, Cleasby ME. Palmitoleic acid prevents palmitic acid-induced macrophage activation and consequent p38 MAPK-mediated skeletal muscle insulin resistance. *Mol Cell Endocrinol*. 2014; **393**:129-42. doi: 10.1016/j.mce.2014.06.010. Epub Jun 26.
10. Chan KL, Pillon NJ, Sivaloganathan DM, et al. Palmitoleate Reverses High Fat-induced Proinflammatory Macrophage Polarization via AMP-activated Protein Kinase (AMPK). *J Biol Chem*. 2015; **290**:16979-88. doi: 10.1074/jbc.M115.646992. Epub 2015 May 18.
11. Bolsoni-Lopes A, Festuccia WT, Farias TS, et al. Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARalpha-dependent manner. *Am J Physiol Endocrinol Metab*. 2013; **305**:E1093-102. doi: 10.152/ajpendo.00082.2013. Epub 2013 Sep 10.
12. Zapolska-Downar D, Siennicka A, Kaczmarczyk M, Kolodziej B, Naruszewicz M. Butyrate inhibits cytokine-induced VCAM-1 and ICAM-1 expression in cultured endothelial cells: the role of NF-kappaB and PPARalpha. *J Nutr Biochem*. 2004; **15**:220-8.
13. Sarabi M, Vessby B, Millgard J, Lind L. Endothelium-dependent vasodilation is related to the fatty acid composition of serum lipids in healthy subjects. *Atherosclerosis*. 2001; **156**:349-55.
14. Staiger K, Staiger H, Weigert C, Haas C, Haring HU, Kellerer M. Saturated, but not unsaturated, fatty acids induce apoptosis of human coronary artery endothelial cells via nuclear factor-kappaB activation. *Diabetes*. 2006; **55**:3121-6.
15. Daynes RA, Jones DC. Emerging roles of PPARs in inflammation and immunity. *Nat Rev Immunol* 2002; **2**:748-59.
16. Calder PC. Omega-3 fatty acids and inflammatory processes. *Nutrients*. 2010; **2**:355-74. doi: 10.3390/nu2030355. Epub 2010 Mar 18.
17. Allam-Ndoul B, Guenard F, Barbier O, Vohl MC. Effect of n-3 fatty acids on the expression of inflammatory genes in THP-1 macrophages. *Lipids Health Dis*. 2016; **15**:69. doi: 10.1186/s12944-016-0241-4.
18. Nakanishi A, Tsukamoto I. n-3 polyunsaturated fatty acids stimulate osteoclastogenesis through PPARgamma-mediated enhancement of c-Fos expression, and suppress osteoclastogenesis through PPARgamma-dependent inhibition of NFkB activation. *J Nutr Biochem*. 2015; **26**:1317-27. doi: 10.1016/j.jnutbio.2015.06.007. Epub Jul 26.
19. Chang HY, Lee HN, Kim W, Surh YJ. Docosahexaenoic acid induces M2 macrophage polarization through peroxisome proliferator-activated receptor gamma activation. *Life Sci*. 2015; **120**:39-47. doi: 10.1016/j.lfs.2014.10.014. Epub Oct 31.
20. Wan J, Benkdane M, Teixeira-Clerc F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. *Hepatology*. 2014; **59**:130-42. doi: 10.1002/hep.26607. Epub 2013 Nov 20.
21. Galvan-Pena S, O'Neill LA. Metabolic reprogramming in macrophage polarization. *Front Immunol*. 2014; **5**:420. doi: 10.3389/fimmu.2014.00420. eCollection 2014.
22. Palsson-McDermott EM, Curtis AM, Goel G, et al. Pyruvate kinase M2 regulates Hif-1alpha activity and IL-1beta induction and is a critical determinant of the warburg effect in LPS-activated macrophages. *Cell Metab*. 2015; **21**:65-80. doi: 10.1016/j.cmet.2014.12.005.
23. Zizzo G, Cohen PL. The PPAR-gamma antagonist GW9662 elicits differentiation of M2c-like cells and upregulation of the MerTK/Gas6 axis: a key role for PPAR-gamma in human macrophage polarization. *J Inflamm (Lond)*. 2015; **12**:36. doi: 10.1186/s12950-015-0081-4. eCollection 2015.
24. Schneider C, Nobs SP, Kurrer M, Rehrauer H, Thiele C, Kopf M. Induction of the nuclear receptor PPAR-gamma by the cytokine GM-CSF is critical for the differentiation of fetal monocytes into alveolar macrophages. *Nat Immunol*. 2014; **15**:1026-37. doi: 10.38/ni.3005. Epub 2014 Sep 28.

25. Jiang C, Ting AT, Seed B. PPAR-gamma agonists inhibit production of monocyte inflammatory cytokines. *Nature*. 1998; **391**:82-6.

26. Ricote M, Li AC, Willson TM, Kelly CJ, Glass CK. The peroxisome proliferator-activated receptor-gamma is a negative regulator of macrophage activation. *Nature*. 1998; **391**:79-82.

27. Rogue A, Spire C, Brun M, Claude N, Guillouzo A. Gene Expression Changes Induced by PPAR Gamma Agonists in Animal and Human Liver. *PPAR Res*. 2010; **2010**:325183.:10.1155/2010/325183. Epub 2010 Oct 19.

28. DeLuca JG, Doebber TW, Kelly LJ, et al. Evidence for peroxisome proliferator-activated receptor (PPAR)alpha-independent peroxisome proliferation: effects of PPARgamma/delta-specific agonists in PPARalpha-null mice. *Mol Pharmacol*. 2000; **58**:470-6.

29. Brun RP, Tontonoz P, Forman BM, et al. Differential activation of adipogenesis by multiple PPAR isoforms. *Genes Dev*. 1996; **10**:974-84.

30. Bolsoni-Lopes A, Festuccia WT, Chimin P, et al. Palmitoleic acid (n-7) increases white adipocytes GLUT4 content and glucose uptake in association with AMPK activation. *Lipids Health Dis*. 2014; **13**:199.:10.1186/476-511X-13-199.

31. Souza CO, Teixeira AA, Biondo LA, Lima EA, Jr., Batatinha HA, Rosa Neto JC. Palmitoleic Acid Improves Metabolic Functions in Fatty Liver by PPARalpha-Dependent AMPK Activation. *J Cell Physiol* 2016; **7**:25715.

32. Duckett SK, Volpi-Lagreca G, Alende M, Long NM. Palmitoleic acid reduces intramuscular lipid and restores insulin sensitivity in obese sheep. *Diabetes Metab Syndr Obes*. 2014; **7**:553-63.:10.2147/DMSO.S72695. eCollection 2014.

33. Zhang Z, Lowry SF, Guarente L, Haimovich B. Roles of SIRT1 in the acute and restorative phases following induction of inflammation. *J Biol Chem*. 2010; **285**:41391-401. doi: 10.1074/jbc.M110.174482. Epub 2010 Oct 21.

34. Zhao X, Zmijewski JW, Lorne E, et al. Activation of AMPK attenuates neutrophil proinflammatory activity and decreases the severity of acute lung injury. *Am J Physiol Lung Cell Mol Physiol*. 2008; **295**:L497-504. doi: 10.1152/ajplung.90210.2008. Epub 2008 Jun 27.

35. Salminen A, Hyttinen JM, Kaarniranta K. AMP-activated protein kinase inhibits NF-kappaB signaling and inflammation: impact on healthspan and lifespan. *J Mol Med (Berl)*. 2011; **89**:667-76. doi: 10.1007/s00109-011-0748-0. Epub 2011 Mar 23.

36. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987; **162**:156-9.

37. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 2001; **25**:402-8.

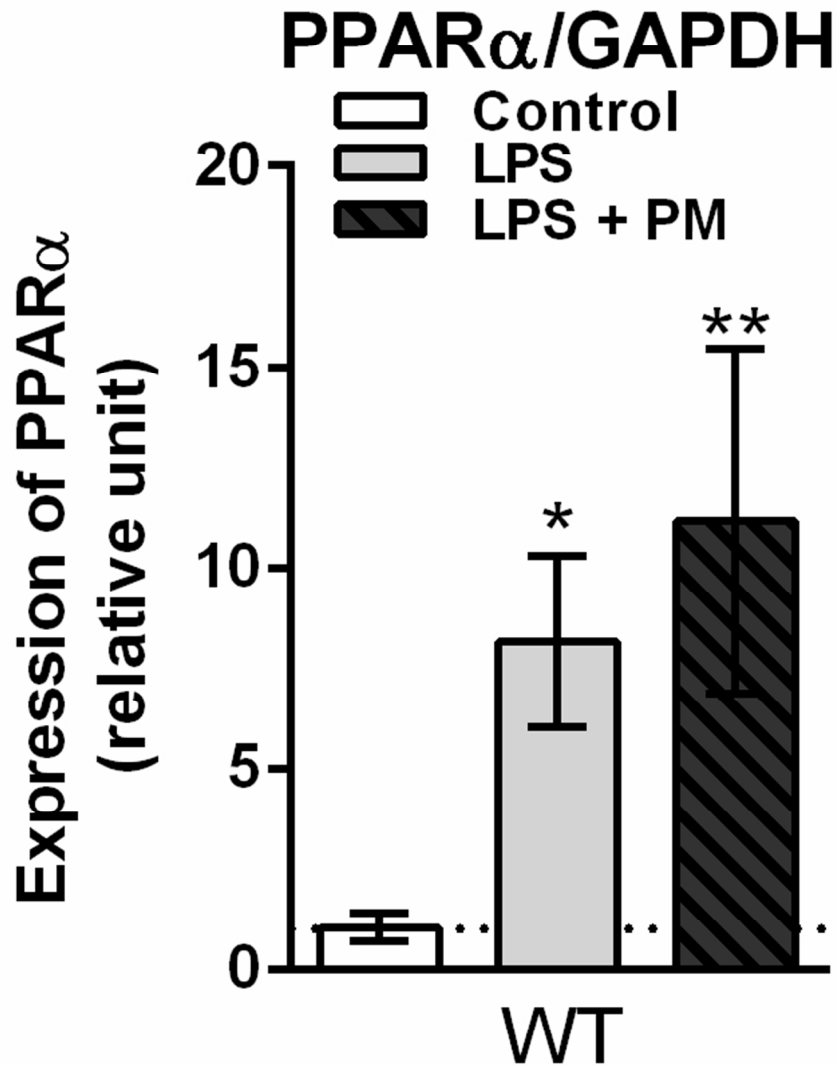


Fig 1. Palmitoleic acid with LPS synergically increased the expression of PPAR α . Relative mRNA expression of peroxisome proliferator-activated receptor α (PPAR α) in macrophages extracted from WT mice and incubated with RPMI (Control), LPS, or palmitoleic acid with LPS (LPS PM) for 24 h. Ct were normalized to glyceraldehyde-3-phosphate (GAPDH). Data are mean \pm SEM of 8 animals. One-way ANOVA followed by Bonferroni, * p <0.05 and ** p <0.01 vs. control.

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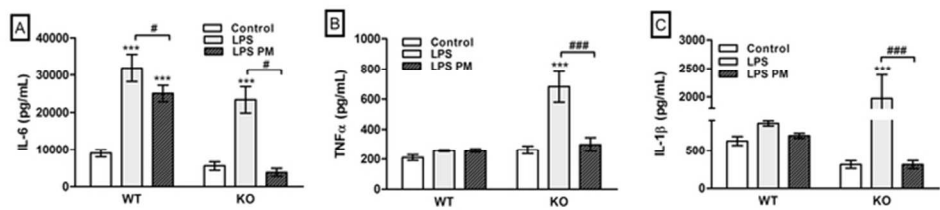


Fig 2. Palmitoleic reduced inflammation stimulated by LPS, independently of PPARα. Concentrations of IL-6 (A), TNF-α (B) and IL-1β (C) produced by macrophages from WT and PPARα KO mice incubated with RPMI (Control), LPS or palmitoleic acid with LPS (LPS PM) for 24 h. Data are mean ± SEM of 8 (WT) or 10 (KO) animals. Two-way ANOVA followed by Bonferroni post test, *p<0.05 and ***p<0.001 vs. control; #p<0.05, ##p<0.01 and ###p<0.001 vs. LPS.

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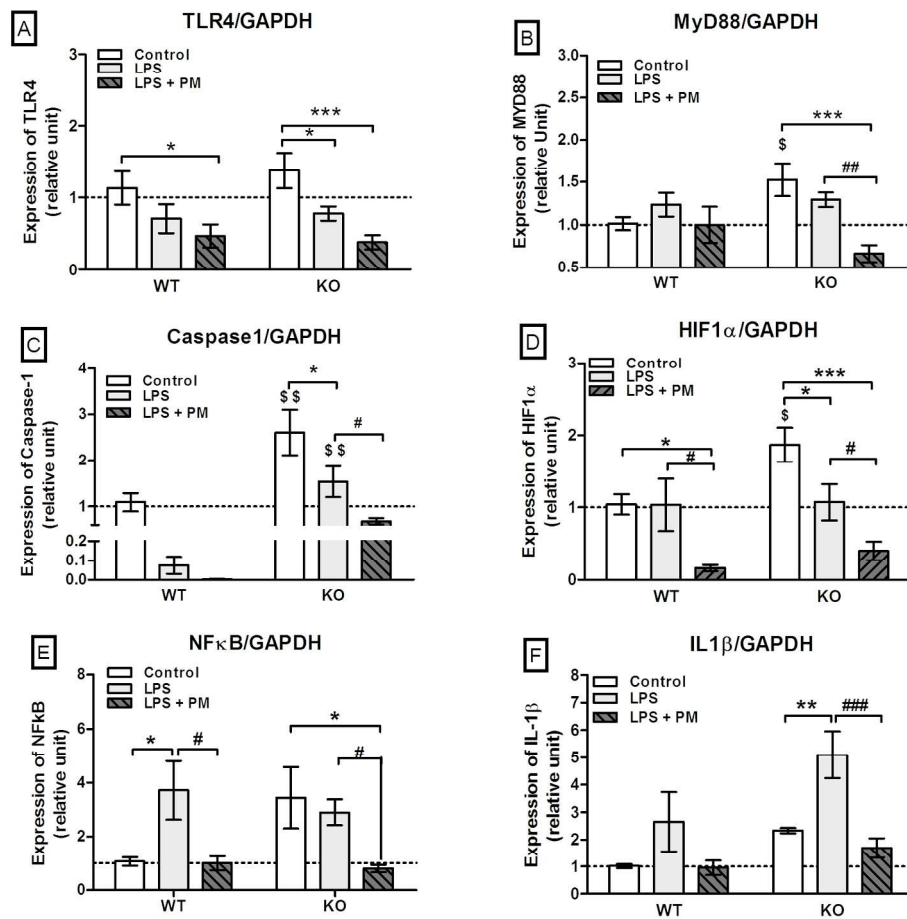


Fig 3. Palmitoleic acid reduced the expression of inflammatory genes independently of PPAR α . Relative mRNA expression of toll Like receptor-4 (TLR4) (A), myeloid differentiation primary response 88 (MyD88) (B), caspase-1 (C), hypoxia-inducible factor- α (HIF1 α) (D), nuclear factor kappa-light-chain-enhancer of activated B cells (NF κ B) (E) and interleukin 1 β (IL-1 β) (F) of WT and KO mice macrophages incubated with palmitoleic acid. Macrophages from WT and PPAR α KO mice incubated with RPMI (Control), LPS or palmitoleic acid (PM) 600 μ M with LPS (LPS PM) for 24 h. All Ct values were normalized to glyceraldehyde-3-phosphate (GAPDH). Data are mean \pm SEM of 6 (WT) or 8 (KO) animals. Two-way ANOVA followed by Bonferroni post test; * p <0.05 and *** p <0.001 vs. control; # p <0.05 vs. LPS; \$ p <0.01 KO vs. respective WT group.

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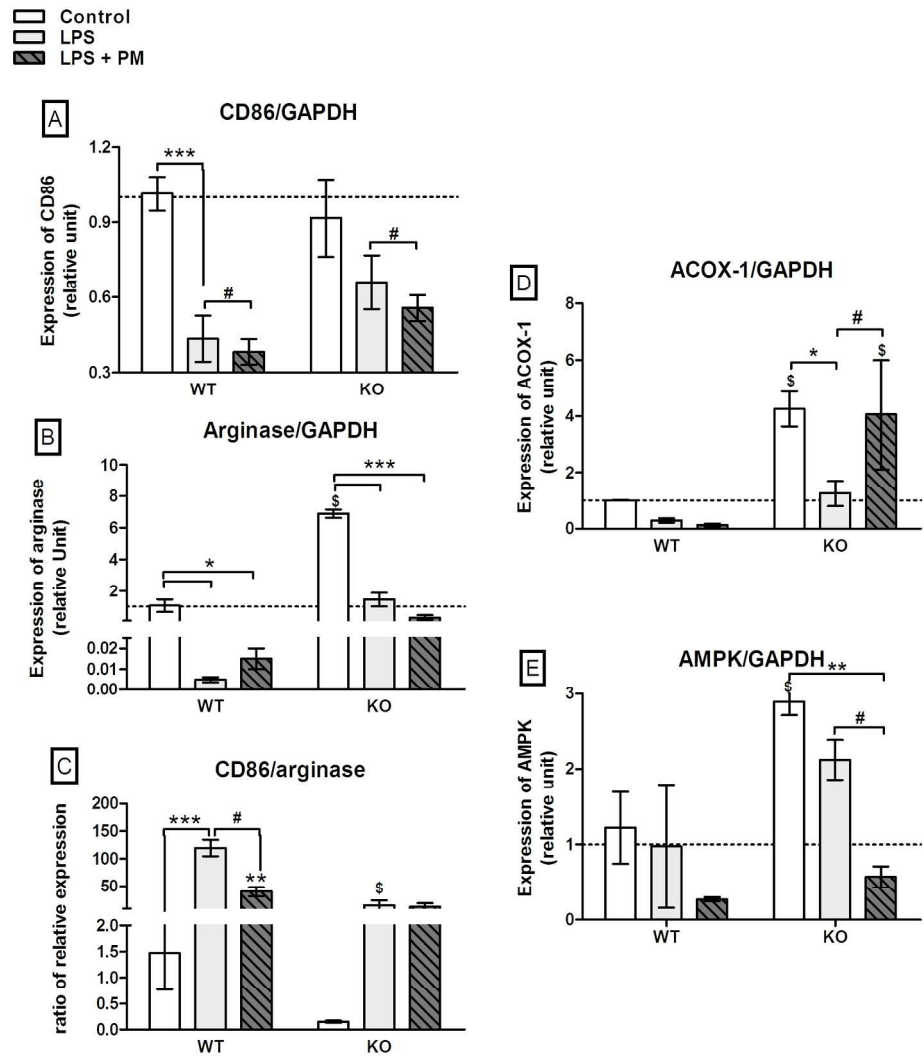


Fig 4. Palmitoleic acid decreased the expressed ratio of M1/M2 in WT and increase pro-oxidative gene ACOX-1 (M2) in KO macrophages. Relative mRNA expression of CD86 (A), and Arginase (B) the ratio of arginase to CD86 (C) and Acyl-CoA oxidase 1 (ACOX-1) (D) and AMP-activated protein kinase (AMPK) (E) of WT and PPARα KO mice macrophages incubated with RPMI (Control), LPS or palmitoleic acid with LPS (LPS PM) for 24 h. All Ct values were normalized to glyceraldehyde-3-phosphate (GAPDH). Data are mean ± SEM of 6 (WT) or 8 (KO) animals. Two-way ANOVA followed by Bonferroni post test; *p<0.05 and ***p<0.001 vs. control; #p<0.05 and ##p<0.01 vs. LPS; \$p<0.01 KO vs. respective WT group.

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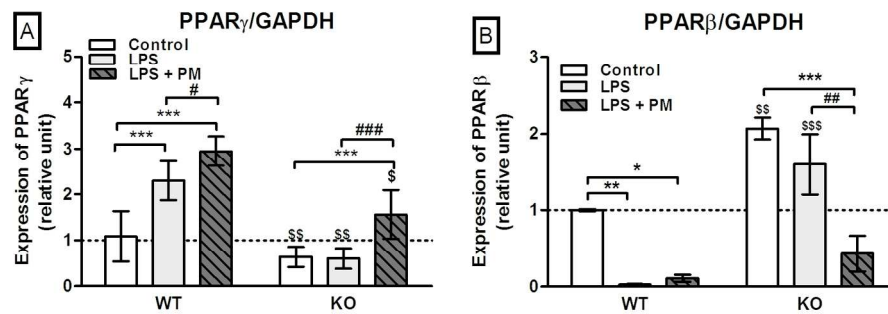


Fig 5. Palmitoleic acid modulated the expression of PPARs differently. Relative mRNA expression of peroxisome proliferator-activated receptor γ (PPAR γ) (A), and PPAR β (B) of WT and PPAR α KO mice macrophages incubated with RPMI (Control), LPS or palmitoleic acid with LPS (LPS PM) for 24 h. All Ct values were normalized to glyceraldehyde-3-phosphate (GAPDH). Data are mean \pm SEM of 6 (WT) or 8 (KO) animals. Two-way ANOVA followed by Bonferroni post test; * $p < 0.05$ and *** $p < 0.001$ vs. control; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. LPS; \$ $p < 0.05$, \$\$ $p < 0.01$ and \$\$\$ $p < 0.001$ KO vs. respective WT group.

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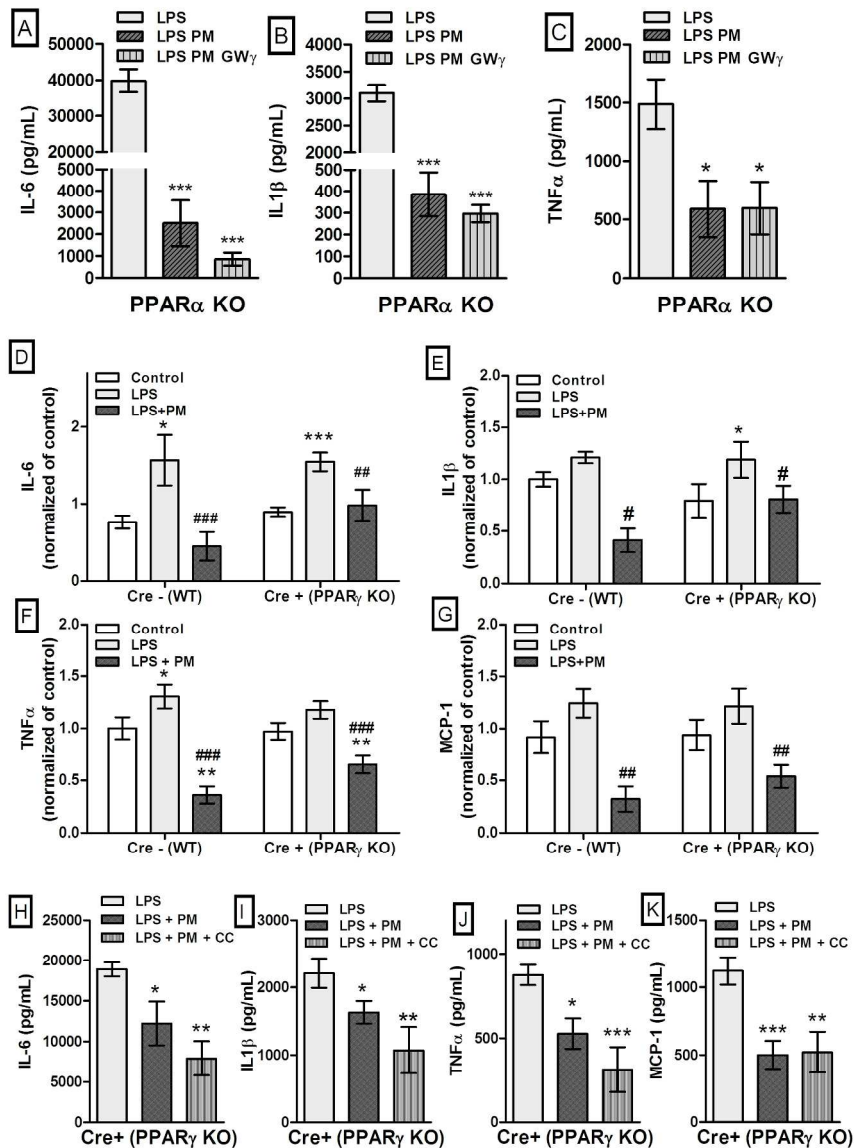


Fig 6. Palmitoleic acid reduced the inflammation induced by LPS independently of PPAR γ and AMPK. Concentrations of IL-6 (A), TNF- α (B) and IL-1 β (C) produced by macrophages from PPAR α KO mice incubated with LPS, palmitoleic acid with LPS (LPS PM) or palmitoleic acid with LPS plus an inhibitor of PPAR γ (GW9662) (LPS PM GW γ) for 24 h (n=6). Concentrations of IL-6 (D), TNF- α (E) IL-1 β (F) and MCP-1 (G) produced by macrophages from WT (Cre-) and PPAR γ KO (Cre+) incubated with RPMI (control), LPS or palmitoleic acid with LPS (LPS PM) for 24 h (n=8 to 12). Concentrations of IL-6 (H), TNF- α (I) IL-1 β (J) and MCP-1 (K) produced by macrophages from PPAR γ KO (Cre+) incubated with LPS, palmitoleic acid with LPS (LPS PM) or palmitoleic acid with LPS plus an inhibitor of AMPK (Compound C) (LPS PM CC) for 24 h (n=7). (A, B, C, H, I, J and K) One way Anova followed by Bonferroni post test; *p<0.05, **p<0.01 and ***p<0.001 vs. control. (D, E, F and G) Two-way ANOVA followed by Bonferroni post test; *p<0.05 and ***p<0.001 vs. control; #p<0.05, ##p<0.01 and ###p<0.001 vs. LPS.

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For Peer Review

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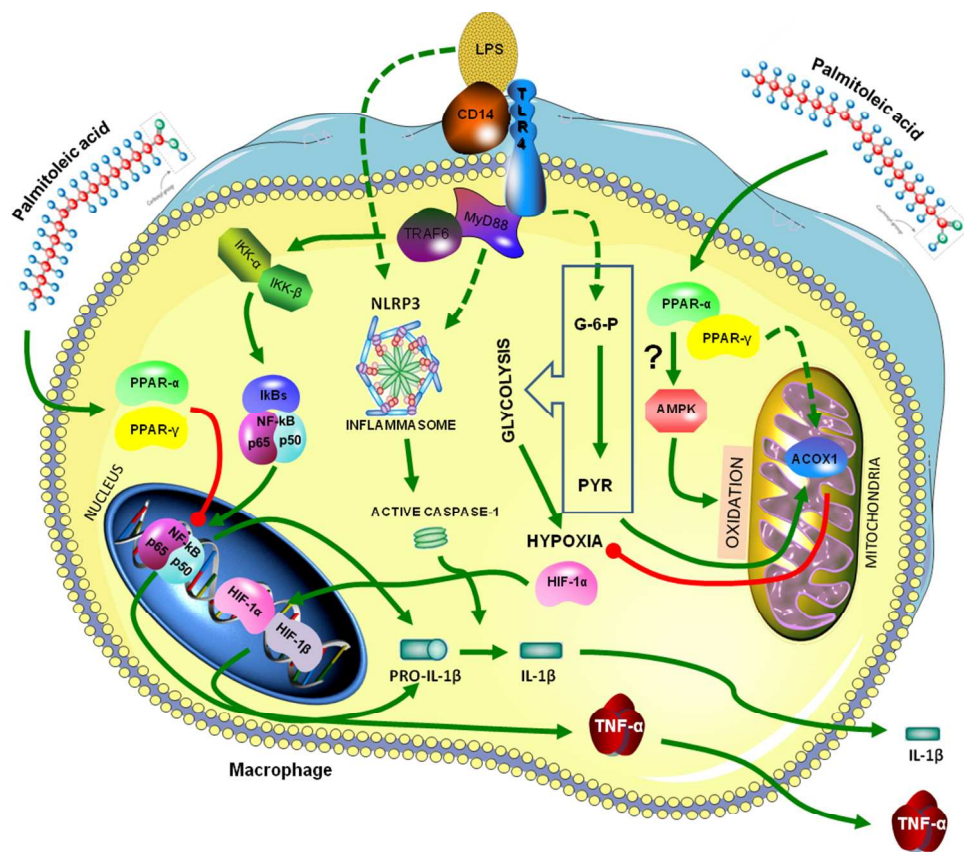


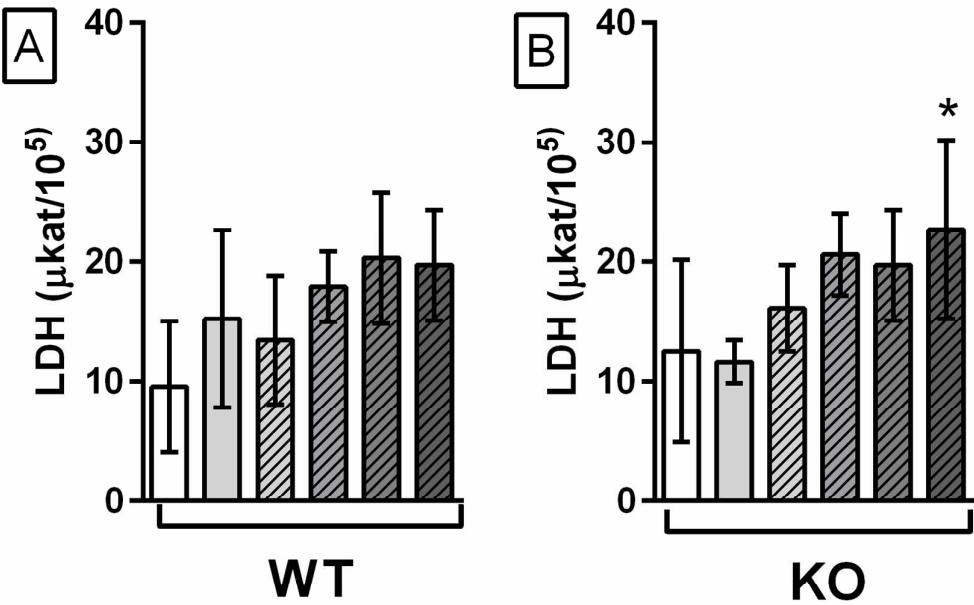
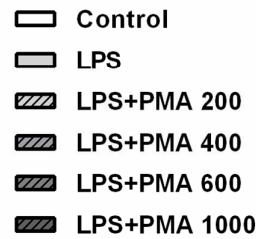
Fig 7. Although palmitoleic acid induced the activation of both, PPAR α and PPAR γ its anti-inflammatory effects are not strictly dependent of those PPARs, but are related to modulation of genes from the inflammasome complex. PPAR α and PPAR γ , upregulated by palmitoleic acid in our study, may act in an anti-inflammatory manner regulating the macrophages differentiation to M2 phenotype and interfering with the NF κ B activation. When activated by LPS stimulus, TLR4 (Toll-Like Receptor-4) triggers a cascade that can be dependent of the adaptors protein MyD88 (Myeloid differentiation primary response gene 88) and leads to phosphorylation of the IKK complex (Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-Cells Kinase), allowing the NF-KB translocation to the nucleus, where it induces the expression of TNF α (Tumor necrosis factor α) and pro-IL1 β (interleukin 1 β), which can be cleaved and activated to IL1 β by caspase-1. TLR4 activation, can also induce a non-canonical activation of IL1 β , which enhances HIF1 α (Hipopxia Induced Factor-1 alpha), switch the metabolism to aerobic glycolysis and promotes the transcription of pro-IL1 β .

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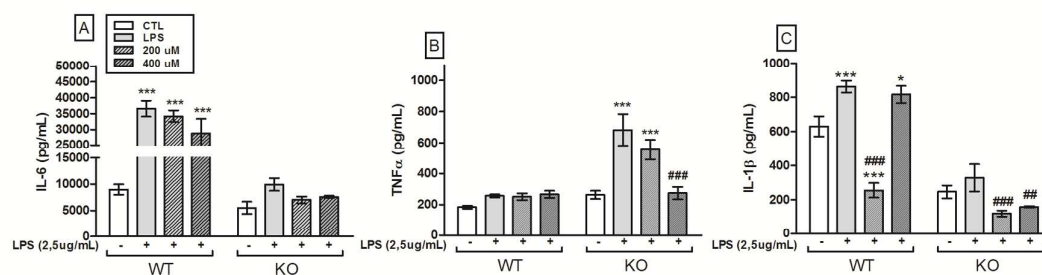
Table 1. Sequences of forward and reverse primers used for qRT-PCR.

Gene	Primer Forward	Primer Reverse
GAPDH	CAAGCTCATTTCTGGTATGACA	GCCTCTCTTGCTCAGTGTCC
PPARα	TGCAATTCGCTTTGGAAGAA	CTTGCCCAGAGATTTGAGGT
TLR4	TCCAGCCACTGAAGTTCT	CAGCAAAGTCCCTGATGA
MyD88	TCCAGGTGTCCAACAGAAG	ACTTGGTGCAAGGGTTGGT
Caspase-1	CACCTCTTTCACCATCTCC	ACATCTTTCTCCGAGGGTT
HIF1α	AGTCAGCAACGTGGAAGGT	CGTCATGGGTGGTTTCTTG
NFκB	CCAAGTGGCAGGTATTTGAC	GCTGCTTCATGTCCCCTTG
IL-1β	TGGGATCCACACTCTCCA	GGAGAACCAAGCAACGACA
CD86	TGTTTCCGTGGAGACGCAAG	TTGAGCCTTTGTAAATGGGCA
Arginase	TCCTCCACGGGCAAATTCC	GCTGGACCATATTCCACTCCTA
PPARγ	TGAAGCTCCAAGAATACCAAAG	GCCATGAGGGAGTTAGAAGG
PPARβ	AGCCACAACGCACCCTTT	ACCAGCTGTTTCCACACCA
ACOX-1	TACGTGCAGCCAGATTGGTA	ACGCCACTTCCTTGCTCTT
AMPK	AGCCGACTTTGGTCTTTCAA	GCCTGCGTACAATCTTCCTG

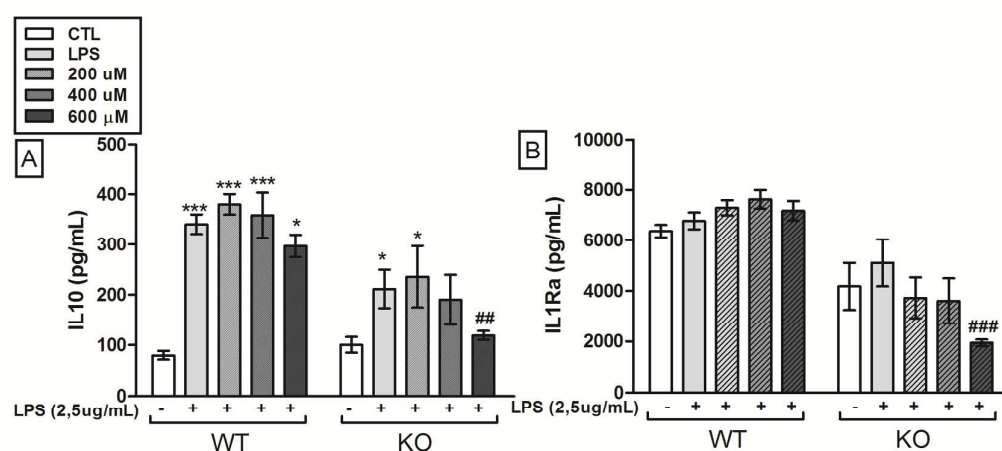
SUPPLEMENTARY DATA



Supp 1. Lactate dehydrogenase (LDH) activity in media of macrophages isolated from the peritoneal cavity of WT (C57BL6) and KO (PPARα knockout) mice and incubated with RPMI (Control), LPS or several concentrations (200 to 1000 μM) of palmitoleic acid plus LPS (LPS+PM) for 24 h. Data are mean ± SEM of 8 (WT) or 10 (KO) animals. One-way ANOVA followed by Bonferroni post test; *p<0,05 vs. control.



Supp 2. Concentrations of IL-6 (A), TNF-α (B) and IL-1β (C) produced by macrophages extracted from WT (C57BL/6) and KO (PPARα knockout) mice, and incubated with RPMI (Control), LPS or several concentrations (200 and 400 μM) of palmitoleic acid plus LPS for 24 h. Data are mean ± SEM of 8 (WT) or 10 (KO) animals. Two way ANOVA followed by Bonferroni post test; * $p < 0.05$ and *** $p < 0.001$ vs. each control; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ vs. LPS.



Supp 3. Concentrations of IL-10 (A) and IL-1Ra (B) produced by macrophages extracted from WT (C57BL/6) and KO (PPARα knockout) mice, and incubated with RPMI (Control), LPS or several concentrations (200 to 600 μM) of palmitoleic acid plus LPS for 24 h. Data are mean ± SEM of 8 (WT) or 9 (KO) animals. Two-way ANOVA followed by Bonferroni; * $p < 0.05$ and *** $p < 0.001$ vs. control, ## $p < 0.01$ and ### $p < 0.001$ vs. LPS.