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**Abstract**

Niacin activates HCA2 receptor coupled to distal enzymes that results in the release of PGD2. However, little is known on PGD2-producing cells and the role of dietary fatty acids in the regulation of PGD2 production. With the exception of neutrophils and monocytes, all cells but notably M-CSF macrophages exhibited a timely dependent PGD2 production upon niacin challenge. Short pre-treatment of M-CSF macrophages with autologous postprandial TRLs induced the down-regulation of HCA2 gene and up-regulation of genes encoding COX1 and COX2 enzymes in a fatty acid-dependent manner. These effects were paralleled by a higher PGD2 production with postprandial TRL-SFAs. The niacin-mediated transcriptional activity of all genes involved in PGD2 biosynthesis was desensitized in a time-dependent manner by postprandial TRLs, leading to a decreased PGD2 release. In vivo, the net excursions of PGD2 in plasma from volunteers in response to the different dietary fatty acids plus a single-dose of immediate-release niacin followed similar fatty acid-dependent patterns as those found for PGD2 release in vitro. M1 and niacin-activated naïve macrophages are major PGD2-producing cells in the human monocyte-macrophage lineage. The predominant fatty acid class in the diet acutely modulates PGD2 biosynthetic pathway both in vitro and in vivo.



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| **Keywords** | Niacin; exogenous fatty acids; postprandial state; prostaglandin D2; myeloid cells |



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## HIGHLIGHTS

1. M1 and niacin-activated naïve macrophages are major $PGD_2$-producing cells in the monocyte-macrophage lineage.
2. $PGD_2$ biosynthetic genes are modulated by postprandial triglyceride-rich lipoproteins in a fatty acid-dependent manner.
3. Niacin-induced $PGD_2$ biosynthesis is desensitized by postprandial triglyceride-rich lipoproteins.
4. Postprandial $PGD_2$ excursions are modulated by the class of dietary fatty acid in healthy subjects.

# Exogenous Fatty Acids and Niacin On Acute Prostaglandin $D_2$ Production In Human Myeloid Cells

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**ABSTRACT**

Niacin activates $HCA_2$ receptor coupled to distal enzymes that results in the release of $PGD_2$. However, little is known on $PGD_2$-producing cells and the role of dietary fatty acids in the regulation of $PGD_2$ production. With the exception of neutrophils and monocytes, all cells but notably M-CSF macrophages exhibited a timely dependent $PGD_2$ production upon niacin challenge. Short pre-treatment of M-CSF macrophages with autologous postprandial TRLs induced the down-regulation of *$HCA_2$* gene and up-regulation of genes encoding COX1 and COX2 enzymes in a fatty acid-dependent manner. These effects were paralleled by a higher $PGD_2$ production with postprandial TRL-SFAs. The niacin-mediated transcriptional activity of all genes involved in $PGD_2$ biosynthesis was desensitized in a time-dependent manner by postprandial TRLs, leading to a decreased $PGD_2$ release. *In vivo*, the net excursions of $PGD_2$ in plasma from volunteers in response to the different dietary fatty acids plus a single-dose of immediate-release niacin followed similar fatty acid-dependent patterns as those found for $PGD_2$ release *in vitro*. M1 and niacin-activated naïve macrophages are major $PGD_2$-producing cells in the human monocyte-macrophage lineage. The predominant fatty acid class in the diet acutely modulates $PGD_2$ biosynthetic pathway both *in vitro* and *in vivo*.

**Keywords:** Niacin; exogenous fatty acids; postprandial state; prostaglandin D2; myeloid cells

## 1. Introduction

Niacin (also commonly known as nicotinic acid or vitamin B3) is well established for the treatment of dyslipidemia [1] and reduction of cardiovascular risk [2]. However, its clinical use has been greatly limited due to an intense skin flush within a few minutes of niacin ingestion [3]. This side effect is mediated by the niacin-induced agonism of hydroxy-carboxylic acid receptor 2 ($HCA_2$) to produce the vasodilator prostaglandin D2 ($PGD_2$) in epidermal Langerhans cells and keratinocytes [4]. $PGD_2$ is also an early-phase mediator in several inflammatory conditions [5] and hypersensitivity reactions such as anaphylaxis [6], with the major contribution from resident mast cells in peripheral tissues [7]. The stimulation of $HCA_2$ by niacin triggers a cascade of distal but functionally linked enzymes that encompasses the cytosolic phospholipase A2 ($PLA_2$) for the release of endogenous arachidonic acid from the cell membrane, the constitutive cyclooxygenase isoform 1 (COX1) and the inducible cyclooxygenase isoform 2 (COX2) as the rate-limiting steps for the conversion of arachidonic acid into the unstable cyclic endoperoxide $PGH_2$, and the PGD synthase (PTGDS) for the isomerization of $PGH_2$ to yield $PGD_2$ [8]. $PGD_2$ can be non-enzymatically dehydrated to form 15-deoxy-$\Delta^{12,14}$ prostaglandin J2 (15d-$PGJ_2$), the most potent endogenous ligand of peroxisome proliferator-activated receptor $\gamma$ (PPAR$\gamma$) that drives the feedback regulation of gene encoding $HCA_2$ [9]. The PPARs are promiscuous in terms of their interaction with ligands and they also exhibit distinctive affinity for selective fatty acids, including saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and long-chain polyunsaturated fatty acids (PUFAs) of the omega-3 family exogenously supplied by postprandial triglyceride-rich lipoproteins (TRLs) [10]. These

observations raise the issue of whether the acute metabolism of dietary fatty acids might be linked to signaling mechanism for niacin-induced $PGD_2$ production.

The full-length cDNA of $HCA_2$ was first cloned from a cDNA library of human monocytes [11] but other cells of innate immune system (neutrophils and antigen-presenting cells derived from $CD34^+$ umbilical blood precursor cells) also expressed $HCA_2$ [12,13]. The physiological role of $HCA_2$ and its contribution to niacin-induced $PGD_2$ production in monocytes and neutrophils is unclear. However, primary peritoneal macrophages from patients subjected to paracentesis and macrophage-like THP-1 cells following treatment with phorbol-12-myristate-13-acetate have been shown to produce $PGD_2$ by a COX-dependent mechanism in response to niacin [14]. These data suggest that the niacin-$HCA_2$ axis is usable for $PGD_2$ production in the myeloid-differentiated lineage. In different tissue environments, monocyte myeloid cells can mature and differentiate into macrophages and dendritic cells (DCs) [15]; macrophages can further undergo polarization into M1 classical macrophages by Th1 mediators and into M2 alternative macrophages by Th2 mediators [15]. Little is known regarding the niacin-induced $PGD_2$ production in these cells.

In the present study, we sought first to determine the competence of primary monocytes, neutrophils, and four monocyte/macrophage-derived subsets (M-CSF macrophages as referred to only differentiated in M-CSF but not polarized macrophages, M1, M2 macrophages, and DCs) to produce $PGD_2$ upon niacin challenge. In M-CSF macrophages, which were the major niacin-

induced $PGD_2$-producing myeloid subset, the influence of niacin and postprandial TRLs with different class of predominant fatty acids (SFAs, MUFAs or omega-3 long-chain PUFAs) on the gene expression level and function of $HCA_2$ and downstream signaling enzymes for $PGD_2$ synthesis was also investigated.

## 2. Materials and methods

This study was conducted according to Good Clinical Practice Guidelines and in line with the principles outlined in the Helsinki Declaration of the World Medical Association. Informed consent for the study was obtained (University of Seville, AGL2011-29008).

### *2.1. Postprandial TRL isolation*

Oral fat emulsions were prepared according to the method described by our Patent WO/2014/191597. They consist of water, sucrose, fat (50 g/ $m^2$ body surface area), emulsifier, and flavoring. Six volunteers, aged 25 to 35 years, non-smokers, with no medical history of disease known, abnormality of hematological or biochemical parameters were recruited. After an overnight fasting period of 12 h, all of them were given, over three different occasions, oral fat emulsions containing cow's milk cream (meal rich in SFAs), refined olive oil (meal rich in MUFAs) or refined olive plus a dose of omega-3 long-chain PUFAs, which consisted of 920 mg of eicosapentaenoic acid (EPA) and 760 mg of docosahexaenoic acid (DHA) (meal rich in MUFAs + omega-3 long-chain PUFAs). At the postprandial lipemic peak, i.e. 2-3 h following the ingestion of the oral fat emulsion, venous blood was collected into $K_3$EDTA-containing

Vacutainer tubes (Becton Dickinson, NJ, USA). TRLs were isolated, pooled, and dialyzed against cold PBS [16]. TRLs were then immediately stored at -80 ºC. Lipid oxidizability of postprandial TRL was checked (TBARS level) during isolation and storage, but oxidation of lipids was not detected. TRLs were tested for LPS contamination using the Pierce LAL Chromogenic Endotoxin Quantification kit (Thermo Scientific, Madrid, Spain). LPS contamination was always <0.2 EU/mL. Triglyceride concentration in postprandial TRLs was determined by colorimetric assay kit TG GPO-POD (Bioscience Medical, Madrid, Spain).

*2.2. Fat and TRL fatty acid composition*

The fatty acid composition of cow's milk cream, refined olive oil, and refined olive oil plus omega-3 long-chain PUFAs was determined, in triplicate from the same lot, by the method described in EEC/796/2002 [17] using a gas chromatography system (HP-5890, Hewlett-Packard) equipped with flame ionization detector and a SP-2380 capillary column (Supelco, 30 m $\times$ 0.32 mm) packed with cyanopropyl siloxane (0.25 μm) (**Table 1**). The initial column temperature was 165 ºC, which was held for 10 min, then programmed from 165 ºC to 200 ºC at 1.5 ºC/min. Injector and detector temperature were 250 ºC, with the carrier gas $H_2$. For fatty acid composition in postprandial TRLs (named TRL-SFAs from cow's milk cream, TRL-MUFAs from refined olive oil, and TRL-MUFAs+ω3 from refined olive oil plus omega-3 long-chain PUFAs), aliquots of 100 μL were lyophilized. A solution composed of 2.64 mL of methanol:toluene:dimethoxypropane:sulphuric acid (16.5:5:1:1) and heptane was added on the lyophilized residue. After shaking, and incubating the mixture

at 80 °C for 1 h, the upper phase was transferred to another vial and dried with a stream of $N_2$ gas. The resulting extract was dissolved in heptane and the fatty acid methyl esters were analyzed into a gas chromatography system as described above (**Table 2**).

*2.3. Monocyte and neutrophil isolation*

The same six volunteers who took part as donors of postprandial TRLs participated as donors of leukocytes. After an overnight fasting period of 12 h, peripheral blood samples were drawn from a large antecubital vein and collected into $K_3$EDTA-containing tubes (BD). Peripheral blood mononuclear cells (MNCs) were isolated by centrifugation over a Ficoll-Histopaque (Sigma, Madrid, Spain) gradient. Monocytes were isolated from peripheral blood MNCs using CD14 microbeads and LS columns on a midiMACS system (Miltenyi Biotec, Madrid, Spain). Neutrophils were isolated by dextran sedimentation (2% dextran/0.9% NaCl) (Sigma) from the fraction of peripheral blood polymorphonuclear cells (PMNCs). Residual erythrocytes were removed using hypotonic lysis with 0.2% and 1.6% saline solutions. Monocyte ($CD14^+$) and neutrophil ($CD16^+$) purity was routinely >90% by flow cytometry analysis (FACScanto II flow cytometer and FACSDiva software, BD) and cell viability >95% by trypan blue exclusion (Sigma). The monocytes and neutrophils were seeded at a density of $5\times10^5$ cells/mL and $3\times10^6$ cells/mL, respectively, and cultured in ultra low attachment flasks in RPMI 1640 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-inactivated fetal bovine serum (complete culture medium). $PGD_2$ was determined in the medium of monocytes and neutrophils after 30 min.

*2.4. Monocyte differentiation and polarization*

Monocytes were induced to differentiate for 6 days in the presence of human recombinant M-CSF (50 ng/mL) to obtain naïve (M-CSF) macrophages. Complete culture medium was replaced every 2 days with fresh medium and the cytokine. Degree of differentiation of the resulting population was determined for CD68 antigen using anti-human CD68 monoclonal antibody (Miltenyi Biotec) by flow cytometry analysis (more than 95% of cells were positive for CD68). A similar procedure but using human recombinant GM-CSF (50 ng/mL) and IL-4 (20 ng/mL) was follows to obtain DC-like cells from monocytes. For M1 or M2 polarization, M-CSF macrophages were exposed to LPS (100 ng/mL) and IFNγ (20 ng/mL) or to IL-4 (20 ng/mL), respectively, for additional 24 h. Gene expression levels of markers for DC (CD209 and CD1a), M1 (CD80 and CD64), and M2 (MRC1 and CD200R) were determined by RT-qPCR.

*2.5. Exposure conditions*

Niacin was prepared as an aqueous stock solution further diluted into media without serum. In 24 well plates, cells were exposed to niacin (100 nM) for different times (0–180 min) in complete culture medium (1 mL/well). This niacin concentration induced maximum $PGD_2$ release after exposure of cells to varying doses of niacin (10 nM-100 μM) (data not shown). In some experiments, cells were pre-incubated with aspirin (ASA, 100 μM, 30 min) or postprandial TRLs (TRL-SFAs, TRL-MUFAs and TRL-MUFAs+ω3, 100 μg TG/mL, 30 min) prior to niacin exposure. Cells were treated with postprandial TRLs from the

same volunteer (autologous interaction) and each experiment was done in triplicate. The experiments were conducted with only once thawed postprandial TRL samples. $PGD_2$ was measured in cell culture supernatants.

*2.6. Media LPS and mycoplasma contamination*

All media were tested for LPS contamination using the Pierce LAL Chromogenic Endotoxin Quantification kit. LPS contamination was always <0.2 EU/mL. All cells were tested and free of mycoplasma contamination (Neuron Bio, Granada, Spain).

*2.7. $PGD_2$ assay*

$PGD_2$ was measured using a commercially available competitive enzyme immunoassay kit (Cayman Chemical, MI, USA). Because $PGD_2$ is relatively unstable, all samples underwent a chemical conversion to the stable $PGD_2$ methoxime ($PGD_2$-MOX) prior to assay and according to manufacturer's instructions. Samples were then incubated overnight at 4 °C in 96 well plates pre-coated with mouse monoclonal anti-rabbit IgG, in the presence of rabbit $PGD_2$-MOX antibody and a $PGD_2$-MOX-linked acetylcholinesterase tracer. The complex of $PGD_2$-MOX (either free or tracer linked) and rabbit antibody binds to the pre-coated plates. Following washing, a solution containing acetylcholinesterase reagent was added to wells and plates were read at 412 nm.

*2.8. RNA isolation and qRT-PCR analysis*

Total RNA was extracted by using Trisure Reagent (Bioline). RNA quality was assessed by $A_{260}/A_{280}$ ratio in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Briefly, RNA (1 µg) was subjected to reverse transcription (iScript, Bio-Rad, Madrid, Spain). An amount of 20 ng of the resulting cDNA was used as a template for real-time PCR amplifications. The mRNA levels for specific genes were determined in a CFX96 system (Bio-Rad). For each PCR reaction, cDNA template was added to Brilliant SYBR green QPCR Supermix (Bio-Rad) containing the primer pairs for either gene or for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine phosphoribosyltransferase (HPRT) as housekeeping genes (Table 3). All amplification reactions were performed in triplicate and average threshold cycle (Ct) numbers of the triplicates were used to calculate the relative mRNA expression of candidate genes. The magnitude of change of mRNA expression for candidate genes was calculated by using the standard $2^{-(\Delta\Delta Ct)}$ method. All data were normalized to endogenous reference (GAPDH and HPRT) gene content and expressed as percentage of controls.

*2.9. Acute response to dietary fats and niacin*

The same six volunteers who took part as donors of postprandial TRLs or leukocytes participated in this part of the study. After an overnight fasting period of 12 h, all of them were given oral fat emulsions as indicated above and a single dose of 2 g immediate-release niacin (Twinlab, UT, USA). Venous blood was collected into $K_3$EDTA-containing Vacutainer tubes (BD) at time 0, 30, and 60 min. $PGD_2$ was measured in plasma samples.

*2.10. Data analysis*

All values are expressed as means ± standard deviations (SD). Data were evaluated with Graph Pad Prism Version 5.01 software (San Diego, CA, USA). Area under the curve (AUC) and incremental AUC (iAUC) (area above baseline) were calculated using the trapezoidal rule. The statistical significance of any difference in each parameter among the groups was evaluated by repeated measure one-way analysis of variance (ANOVA), following Tukey's or Dunnett's test for multiple comparisons. *P* values less than 0.05 were considered statistically significant.

## 3. Results

*3.1. Monocytes and monocyte-derived macrophages and dendritic cells but not neutrophils release prostaglandin $D_2$*

The analysis of $PGD_2$ in the medium of monocytes and neutrophils obtained from fasting peripheral blood samples of healthy volunteers is shown in **Fig. 1A**. While monocytes exhibited the capacity for $PGD_2$ release (138 ± 29 pg/mL), neutrophils did not liberate $PGD_2$ into the medium. Monocytes have been shown to differentiate into macrophages or DC-like cells when exposed to M-CSF alone or GM-CSF plus IL-4. In addition, M-CSF macrophages can be polarized into M1 classically activated macrophages by a further treatment with IFNγ plus LPS or into M2 alternatively activated macrophages with IL-4. Therefore, we investigated $PGD_2$ release in all of these monocyte-derived cells (**Fig. 1B**). Gene expression of phenotypic markers for M1 and M2 macrophages, and DCs is shown in **Fig. 1C**. In comparison with M-CSF macrophages, M1 macrophages overexpressed CD80 and CD64 genes, M2

macrophages overexpressed MRC1 and CD200R genes, and DCs overexpressed CD209 and CD1a genes. The medium of M1 macrophages had the largest amount of $PGD_2$ and media of M2 macrophages and DCs the lowest, while $PGD_2$ measured in the medium of M-CSF macrophages was in between (**Fig. 1D**). These observations unveil human primary monocytes and monocyte-derived cells, notably M1 macrophages, as producers of $PGD_2$.

*3.2. M-CSF macrophages are major producers of prostaglandin $D_2$ in response to niacin*

To explore the potential of niacin to induce $PGD_2$ release in these cells, monocytes, neutrophils, M-CSF, M1 and M2 macrophages, and DCs were treated with niacin for a period of 180 min. Monocytes (**Fig. 2A**) and neutrophils (**Fig. 2B**) were not responsive to niacin. However, M-CSF (**Fig. 2C**), M1 (**Fig. 2D**) and M2 (**Fig. 2E**) macrophages, and to a lesser extent DCs (**Fig. 2F**) liberated $PGD_2$ in a time-dependent manner with a peak at 30 min. The iAUC for $PGD_2$ was $365 \pm 13$ pg/mL/min for M-CSF macrophages, $239 \pm 9$ pg/mL/min for M1 macrophages, $224 \pm 6$ pg/mL/min for M2 macrophages, and $82 \pm 8$ pg/mL/min for DCs. Furthermore, the cells with the highest peak response were M-CSF macrophages, followed by M1 and M2 macrophages, and then DCs (**Fig. 2G**). These findings unveil M-CSF macrophages from human primary monocytes as major $PGD_2$-producing cells in response to niacin.

*3.3. Postprandial triglyceride-rich lipoproteins modulate prostaglandin $D_2$ pathway genes and release in niacin-treated M-CSF macrophages*

To investigate the mechanisms by which niacin induced $PGD_2$ release in M-CSF macrophages, we analyzed the expression levels of inducible genes involved in niacin signaling to $PGD_2$ production and release. By this approach, the potential crosstalk between niacin and postprandial TRLs was also explored. These lipoproteins were obtained after the ingestion of standardized meals only differing in the fatty acid composition —mainly containing SFAs (TRL-SFAs), MUFAs (TRL-MUFAs) or MUFAs + omega-3 long-chain PUFAs (TRL-MUFAs+ω3)— from the same volunteers from which circulating monocytes, to further derive M-CSF macrophages, were obtained. As observed with the non-steroidal anti-inflammatory drug and COX inhibitor aspirin, the transcriptional activity of *HCA2* gene was diminished by a pre-treatment for 30 min with postprandial TRLs in M-CSF macrophages (**Fig. 3A**). This effect was similar between the different postprandial TRLs but it was more potent than that induced by aspirin. The pre-treatment of M-CSF macrophages with aspirin also diminished *PLA2G4D* (**Fig. 3B**), *PTGS1* (**Fig. 3C**), and *PTGS2* (**Fig. 3D**) gene expression, which was in part expected [18], while postprandial TRLs did not change mRNA levels of *PLA2G4D* gene but increased those of *PTGS1* and *PTGS2* genes (TRL-SFAs > TRL-MUFAs = TRL-MUFAs+ω3). The expression of *PTGDS* gene was not affected by any pre-treatment (**Fig. 3E**). The addition of niacin to these pre-treated cells provoked time-dependent gene expression changes of *HCA2* (**Fig. 3F**). The mRNA levels of *HCA2* gene peaked at 30 min in the presence of aspirin or postprandial TRLs but were sharply decreased in non-pre-conditioned cells. Niacin-induced kinetics of mRNA expression for *PLA2G4D* gene in cells pre-treated with aspirin or postprandial TRLs resembled those observed for *HCA2* gene (**Fig. 3G**). However, mRNA levels of *PLA2G4D*

gene peaked at 60 min with a larger response in niacin-treated but non-pre-conditioned cells. These transient mRNA profiles were similar for *PTGS1* gene (**Fig. 3H**) but more pronounced for *PTGS2* (**Fig. 3I**) and *PTGDS* (**Fig. 3J**) genes. In further experiments, the pre-treatment of M-CSF macrophages with postprandial TRL-SFAs did not change mRNA levels of *PPARG* (**Fig. 4A**) or its related genes *NAMPT* (**Fig. 4B**) and *SIRT1* (**Fig. 4C**) [19,20], which were increased with TRL-MUFAs and notably TRL-MUFAs+ω3. After the addition of niacin, *PPARG* gene expression was increased over time with a peak at 30 min in non-pre-conditioned cells (**Fig. 4D**). This effect was particularly strong in cells pre-treated with aspirin but less intense in cells pre-treated with postprandial TRL-MUFAs and TRL-MUFAs+ω3. The peak was even prevented in cells pre-treated with postprandial TRL-SFAs. Niacin also induced a maximum in the kinetic of mRNA expression for *NAMPT* gene at 60 min in non-pre-conditioned cells and in pre-treated cells with aspirin, and at 30 min in pre-treated cells with TRL-SFAs or TRL-MUFAs (**Fig. 4E**). However, *NAMPT* gene expression was almost unaffected in pre-treated cells with TRL-MUFAs+ω3. The mRNA levels of *SIRT1* gene peaked at 60 min in non-pre-conditioned cells but at 30 min in all of pre-treated cells (**Fig. 4F**). $PGD_2$ release was further analyzed (**Fig. 5A**). The pre-treatment of M-CSF macrophages with postprandial TRLs increased the amount of $PGD_2$ in the medium (TRL-SFAs > TRL-MUFAs = TRL-MUFAs+ω3). Niacin did not further increase over time $PGD_2$ release in cells pre-treated with postprandial TRLs, while induced a rise with a peak at 30 min in non-pre-conditioned cells (**Fig. 5B**). The administration of a meal with no fat and a single-dose of immediate-release niacin resulted in a time-dependent increase of $PGD_2$ in plasma from the volunteers during the postprandial period (**Fig. 5C**).

This effect was dramatically enhanced by the inclusion and modulated by the fatty acid composition of dietary fatty acids in the meals (meal rich in SFAs > meal rich in MUFAs = meal rich in MUFAs + omega-3 long-chain PUFAs). These data unveil postprandial TRLs as modulators in a fatty-acid-dependent manner of $PGD_2$ pathway genes and release in niacin-treated M-CSF macrophages. They also underscore the physiological relevance of dietary fatty acids on post-meal circulating levels of $PGD_2$.

## 4. Discussion

$PGD_2$ is a lipid mediator that plays a pivotal role in the regulation of several biological processes such as vasodilatation and inflammation. At sites of inflammation, $PGD_2$ is taken up by surrounding cells through G protein-coupled receptors [21] that mediate opposing signals in the promotion and resolution of the inflammatory response [22]. Therefore, the mechanisms involved in $PGD_2$ production deserve particular attention. While $PGD_2$ is predominantly produced *de novo* by mast cells and in lower amounts by basophils, recent studies have reported that macrophages from cryopreserved human monocytes following differentiation in M-CSF may also secrete $PGD_2$ in response to TNFα, remaining a background level of $PGD_2$ release in the absence of stimulus [23]. Here, we found that human primary monocytes and subsets thereof, such as M-CSF macrophages (naïve cells not yet exposed to signals that promote activation and functional polarization), M1 and M2 polarized macrophages, and DC-like cells also produce $PGD_2$. This metabolic ability of monocyte-derived cells was enhanced as monocytes were induced to differentiate, suggesting that $PGD_2$ synthesis is a conserved metabolic pathway in the monocyte/macrophage

lineage and that $PGD_2$ may play a role in the function of the innate immune system. The higher $PGD_2$ release in M1 than in M2 macrophages further indicates the major involvement of $PGD_2$ in M1-mediated processes. In the spectrum of macrophage phenotypes and functions [15], M1 macrophages are mainly associated with chronic inflammation and impaired wound healing in diseases such as atherosclerosis [24], obesity and diabetes [25], glomerulonephritis [26], and encephalitis [27]. In representing the other extreme in a universe of diversity, M2 macrophages are often described as having anti-inflammatory or reparative functions but their persistence can also induce pro-inflammatory disorders such as cystic fibrosis [28] and asthma [29]. $PGD_2$ has been shown to mediate these macrophage plasticity-dependent pathologies [5,30]. As $PGD_2$ release was not detected in neutrophils, it is probably that neutrophil-committed progenitors in the bone marrow lack the expression of any critical element for $PGD_2$ biosynthesis during maturation. The observation that DCs produced $PGD_2$, as much as M2 and M-CSF macrophages, is in accordance with earlier identification of DCs as responsible for $PGD_2$ detection in rat tissues [31] and argue for an effect of $PGD_2$ in dictating the acquisition of immunostimulatory capacity by tissue-resident DCs [32].

Previous studies have shown the protective effect of $HCA_2$ activation in raising $PGD_2$ levels in animal tissues [33,34]. Niacin is a powerful stimulator of $HCA_2$ [4]. We found that niacin treatment transiently increased $PGD_2$ production in human monocyte-derived cells but not in monocytes or neutrophils. DCs were the less and M-CSF macrophages were the most responsive cells to niacin. These observations suggest a new concept by which macrophage subsets and DCs are regulators of $PGD_2$ levels through $HCA_2$. We further investigated gene

expression levels of $HCA_2$ and enzymes ($PLA_2$, COX1, COX2, and PTGDS) involved in niacin-induced $PGD_2$ production [8] in M-CSF macrophages. While *$HCA_2$* gene expression was rapidly suppressed, *PLA2G4D*, *PTGS1*, *PTGS2*, and *PTGDS* mRNA levels were transiently over-represented in response to niacin, supporting previous evidence of a negative feedback loop on $HCA_2$ following its activation [9,35]. This is also in line with the notion that niacin-induced $HCA_2$ receptor-dependent flushing may be desensitized within minutes [36]. In fasting conditions, the homeostatic function to restore the $HCA_2$-$PGD_2$ axis is mediated by ketone bodies produced by the liver from circulating free fatty acids of adipose tissue origin [37]. This mechanism serves to prevent lipolysis, economizes triglyceride utilization, and maintains energy homeostasis during starvation [38]. It is a paradox that the suppression of lipolysis and free fatty acid influx into the bloodstream is also a characteristic feature of the non-fasting state [39]. In response to the ingestion of a meal, exogenous fatty acids incorporated into postprandial TRLs take on the responsibility of meeting energy needs for providing energy to cells for a positive balance [40]. However, the potential of fatty acids from postprandial TRLs to modulate *$HCA_2$*, *PLA2G4D*, *PTGS1*, *PTGS2* and *PTGDS* gene expression, and $PGD_2$ production was unknown. We found that a short treatment (30 min) of M-CSF macrophages with postprandial TRLs decreased *$HCA_2$* mRNA levels as niacin similarly did, while cells lost their sensitivity to niacin in diminishing *$HCA_2$* mRNA levels over the time. This notion establishes a meaningful foundation for a possible regulatory crosstalk and interference between niacin and fatty acids from postprandial TRLs sensing the $PGD_2$ biosynthetic pathway (**Figure 6**). Interestingly, $PGD_2$-derived 15d-$PGJ_2$ [9] and postprandial TRL-derived fatty

acids [10] are *bona fide* ligands for PPARγ. In addition, due to its promiscuity for different fatty acids, the transcriptional activity of PPARγ can be distinctively modulated by SFAs, MUFAs, and PUFAs [10]. We did not observe differences in the expression of *PLA2G4D* and *PTGDS* genes in M-CSF macrophages upon challenge with postprandial TRLs rich in SFAs, MUFAs or MUFAs + omega-3 long-chain PUFAs, but *PTGS1* and *PTGS2* genes were found to be up-regulated by postprandial TRLs in a fatty acid-dependent manner (TRL-SFAs > TRL-MUFAs > TRL-MUFAs+ω3). Under these conditions, the smoothing of niacin-induced kinetics of mRNA for $PGD_2$ biosynthetic genes and $PGD_2$ release suggests the occurrence of desensitization for $PGD_2$ biosynthetic pathway, while only *PTGS1* and *PTGS2* genes remaining transcriptionally modulated by the class of predominant fatty acids in postprandial TRLs. *PPARG* gene and its closely related *NAMPT* and *SIRT1* genes [19,20] exhibited transcription patterns similar to those seen for *PTGS1* and *PTGS2* genes. These transcriptomic changes and the lower $PGD_2$ release with postprandial TRL-MUFAs or TLR-MUFAs+ω3 than with postprandial TRL-SFAs, together with previous studies on PPARγ stimulation by niacin-mediated 15d-$PGJ_2$ in differentiated human monocytic cell lines [41], suggest that exogenous unsaturated fatty acids, probably via PPARγ, are linked with COX enzymes to fine-tune the fuelling of $PGD_2$ production in human macrophages. According to our findings on increased $PGD_2$ production by postprandial TRL-SFAs and reports on short chain SFAs as powerful activators of human $HCA_2$ [42], we cannot exclude the possible involvement of short-chain SFAs from TRL-SFAs in promoting $PGD_2$ production via this mechanism. Further investigations are needed to clarify these possibilities. Moreover, we observed that $PGD_2$ was dramatically

increased in plasma from volunteers who ate a fatty meal rich in SFAs, MUFAs or MUFAs + omega-3 long-chain PUFAs and received a single dose of immediate-release niacin. Consistent with findings found *in vitro*, the magnitude of postprandial $PGD_2$ responses was dependent on the type of dietary fatty acids ingested (meal rich in SFAs > meal rich in MUFAs > meal rich in MUFAs + omega-3 long-chain PUFAs), but there was little if any desensitization effect. These observations may reflect the net impact of dietary fatty acids in the setting of niacin treatment on $PGD_2$-producing cells and mechanisms other than those we have explored *in vitro*.

Collectively, this study expands the knowledge on $PGD_2$-producing cells and add novel insights into the mechanisms by which dietary fatty acids and niacin are involved in the control of metabolic pathways aimed at the production and release of $PGD_2$. In particular, we demonstrate that primary monocytes and monocyte-derived cells (M-CSF, M1 and M2 macrophages, and DCs) are well equipped with genes for $PGD_2$ synthesis, although they secrete $PGD_2$ depending on the state of cellular differentiation. Those genes encoding COX1 and COX2 may be modulated by postprandial TRLs depending on their predominant fatty acid class (SFAs, MUFAs or MUFAs + omega-3 long-chain PUFAs). M1 macrophages were the major producers and postprandial TRL-SFAs were the major inducers of $PGD_2$. Non-polarized macrophages gain advantage to secrete $PGD_2$ in the presence of niacin, although this process is desensitized in a time-dependent manner by postprandial TRLs. Our observations led to new understanding into the dynamic role of dietary fatty acids for the control of niacin-induced $PGD_2$ production and release by the

innate immune system, which could be of relevance in the management of $PGD_2$-mediated disorders.

## Conflicts of interest

The authors state no conflict of interest.

## Acknowledgments

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## Figure legends

**Figure 1**. Human primary monocytes and monocyte-derived cells, notably M1 macrophages, secrete $PGD_2$. Fasting healthy volunteers (n=6) were donors of peripheral blood mononuclear (MNCs) and polymorphonuclear (PMNCs) cells to isolate monocytes and neutrophils, respectively. Monocytes and neutrophils were incubated for 30 min in complete culture medium. Monocytes were differentiated in M-CSF (50 ng/mL) (naïve or M-CSF macrophages) or in GM-CSF (50 ng/mL) plus IL-4 (20 ng/mL) (DC-like cells) for 6 days. M-CSF macrophages were further induced to polarize in LPS (100 ng/mL) plus IFNγ (20 ng/mL) (M1 macrophages) or in IL-4 (20 ng/mL) (M2 macrophages) for additional 24 h. **A**, $PGD_2$ in monocyte and neutrophil supernatants. *$P$<0.01 *vs* neutrophils. **B**, Derivation and characteristics of monocyte-derived cells. **C**, Gene expression of some known markers of polarized macrophages (*CD80* and *CD64* for M1, *MRC1* and *CD200R* for M2) and DCs (*CD209* and *CD1a*). *$P$<0.01 *vs* M-CSF macrophages. **D**, $PGD_2$ in monocyte-derived cell supernatants. *$P$<0.01 *vs* other cell subset.

**Figure 2**. Human primary monocyte-derived cells, notably M-CSF macrophages, secrete $PGD_2$ in response to niacin. **A-F**, $PGD_2$ in monocyte (**A**), neutrophil (**B**), M-CSF macrophage (**C**), M1 macrophage (**D**), M2 macrophage (**E**), and DC-like cell (**F**) supernatants after niacin (100 nM) treatment for 180 min. **G**, Percentages of $PGD_2$ release *vs* non-treated cells. Each experiment was performed in triplicate. *$P$<0.01 *vs* t=0 min.

**Figure 3**. Niacin and postprandial TRLs modulate the expression of *$HCA_2$* gene and genes involved in $PGD_2$ biosynthesis in human M-CSF macrophages. Healthy volunteers (n=6) from which M-CSF macrophages were obtained, were also donors of postprandial TRLs after the ingestion of a standardized oral fat emulsion containing cow's milk cream (TRL-SFAs), refined olive oil (TRL-MUFAs) or refined olive plus a dose of omega-3 long-chain PUFAs (920 mg EPA and 760 mg DHA) (TRL-MUFAs+ω3). M-CSF macrophages and postprandial TRLs (100 μg TG/mL) from the same volunteer (autologous interaction) were incubated for 30 min. Cells were alternatively incubated with aspirin (100 μM). Thereafter, cells were treated with niacin (100 nM) for 180 min. **A-E**, Gene expression of *$HCA_2$* (**A**), *PLA2G4D* (**B**), *PTGS1* (**C**), *PTGS2* (**D**), and *PTGDS* (**E**) before niacin treatment. **P*<0.01 *vs* control, ***P*<0.01 *vs* aspirin, ****P*<0.01 *vs* other TRLs. **F-J**, Gene expression of *$HCA_2$* (**F**), *PLA2G4D* (**G**), *PTGS1* (**H**), *PTGS2* (**I**), and *PTGDS* (**J**) after niacin treatment. For the sake of clarity, statistical differences are not shown. Each experiment was performed in triplicate.

**Figure 4**. Niacin and postprandial TRLs modulate the expression of *PPARG* gene and related genes *NAMPT* and *SIRT1* in human M-CSF macrophages. Healthy volunteers (n=6) from which M-CSF macrophages were obtained, were also donors of postprandial TRLs after the ingestion of a standardized oral fat emulsion containing cow's milk cream (TRL-SFAs), refined olive oil (TRL-MUFAs) or refined olive plus a dose of omega-3 long-chain PUFAs (920 mg EPA and 760 mg DHA) (TRL-MUFAs+ω3). M-CSF macrophages and postprandial TRLs (100 μg TG/mL) from the same volunteer (autologous

interaction) were incubated for 30 min. Cells were alternatively incubated with aspirin (100 μM). Thereafter, cells were treated with niacin (100 nM) for 180 min. **A-C**, Gene expression of *PPARG* (**A**), *NAMPT* (**B**), and *SIRT1* (**C**) before niacin treatment. **P*<0.01 *vs* control, ***P*<0.01 *vs* aspirin, ****P*<0.01 *vs* other TRLs. **D-F**, Gene expression of *PPARG* (**D**), *NAMPT* (**E**), and *SIRT1* (**F**) after niacin treatment. For the sake of clarity, statistical differences are not shown. Each experiment was performed in triplicate.

**Figure 5**. Postprandial TRLs induce $PGD_2$ release in human M-CSF macrophages and dietary fats induce postprandial $PGD_2$ excursions in a fatty acid-dependent manner. Healthy volunteers (n=6) from which M-CSF macrophages were obtained, were also donors of postprandial TRLs after the ingestion of an standardized oral fat emulsion containing cow's milk cream (TRL-SFAs), refined olive oil (TRL-MUFAs) or refined olive plus a dose of omega-3 long-chain PUFAs (920 mg EPA and 760 mg DHA) (TRL-MUFAs+ω3). M-CSF macrophages and postprandial TRLs (100 μg TG/mL) from the same volunteer (autologous interaction) were incubated for 30 min. Cells were alternatively incubated with aspirin (100 μM). Thereafter, cells were treated with niacin (100 nM) for 180 min. Each experiment was performed in triplicate. In addition, the same volunteers were given a standardized oral fat emulsion containing the above dietary fats (meal rich in SFAs, MUFAs or MUFAs + omega-3 long-chain PUFAs) and a single dose of immediate-release niacin (2 g). **A**, $PGD_2$ in cell supernatants. **P*<0.01 *vs* control, ***P*<0.01 *vs* aspirin, ****P*<0.01 *vs* other TRLs. **B**, Percentages of $PGD_2$ release *vs* non-treated cells.

For the sake of clarity, statistical differences are not shown. **C**, $PGD_2$ in post-meal plasma. **P*<0.01 *vs* control, ***P*<0.01 *vs* other TRLs.

**Figure 6**. Schematic representation of the different actions of niacin and fatty acids in postprandial TRLs in $PGD_2$ production. The scheme summarizes the hypothesis by which niacin supplementation and fatty acids in dietary fats would acutely modulate the biosynthesis of $PGD_2$ in the human monocyte-macrophage lineage. This model does not exclude the participation of additional mechanisms of action for niacin and exogenous fatty acids to achieve their effects on the $PGD_2$ biosynthetic pathway. Abbreviations can be found in the text.

**Table 1.** Fatty acid composition of dietary fats.

| | **Cow's milk cream** | **Refined olive oil** | **Refined olive oil + omega-3 long-chain PUFAs** |
|---|---|---|---|
| **Fatty acid** | **g/100 g of fatty acid** | | |
| 4:0, butyric | 0.83 ± 0.16 | - | - |
| 6:0, caproic | 0.25 ± 0.02 | - | - |
| 8:0, caprylic | 0.61 ± 0.07 | - | - |
| 10:0, capric | 2.47 ± 0.13 | - | - |
| 12:0, lauric | 3.09 ± 0.42 | - | - |
| 14:0, myristic | 10.9 ± 0.91 | - | - |
| 16:0, palmitic | 35.5 ± 0.82 | 20.4 ± 0.89 | 20.5 ± 0.64 |
| 16:1(ω-7), palmitoleic | 3.60 ± 0.32 | 0.97 ± 0.17 | 0.82 ± 0.12 |
| 18:0, stearic | 11.5 ± 0.75 | 5.70 ± 0.11 | 4.49 ± 0.36 |
| 18:1(ω-9), oleic | 25.3 ± 0.71 | 61.9 ± 1.23 | 61.5 ± 0.97 |
| 18:2(ω-6), linoleic | 4.27 ± 0.82 | 7.97 ± 0.65 | 8.04 ± 0.53 |
| 18:3(ω-3), α-linolenic | 0.39 ± 0.05 | 1.04 ± 0.13 | 0.94 ± 0.03 |
| 20:5(ω-3), eicosapentaenoic | - | - | 0.92 ± 0.09 |
| 22:6(ω-3), docosahexaenoic | - | - | 0.72 ± 0.10 |
| Others | 0.96 ± 0.42 | 2.05 ± 1.08 | 2.01 ± 0.88 |

Data are means ± SD (n=3).

**Table 2.** Fatty acid composition of postprandial triglyceride-rich lipoproteins.

| | TRL-SFAs | TRL-MUFAs | TRL-MUFAs+ω3 |
|---|---|---|---|
| **Fatty acid** | **g/100 g of fatty acid** | | |
| 4:0, butyric | 0.22 ± 0.09 | - | - |
| 6:0, caproic | 0.13 ± 0.06 | - | - |
| 8:0, caprylic | 0.36 ± 0.12 | - | - |
| 10:0, capric | 1.42 ± 0.41 | - | - |
| 12:0, lauric | 3.77 ± 1.06 | - | - |
| 14:0, myristic | 9.04 ± 1.55 | - | - |
| 16:0, palmitic | 36.3 ± 2.31 | 11.8 ± 1.97 | 12.1 ± 1.34 |
| 16:1(ω-7), palmitoleic | 1.59 ± 0.08 | 0.88 ± 0.32 | 1.46 ± 0.43 |
| 18:0, stearic | 17.1 ± 1.54 | 5.98 ± 0.93 | 5.62 ± 0.83 |
| 18:1(ω-9), oleic | 22.8 ± 2.03 | 66.4 ± 3.27 | 60.7 ± 2.18 |
| 18:2(ω-6), linoleic | 4.24 ± 1.06 | 8.93 ± 1.27 | 10.1 ± 1.44 |
| 18:3(ω-3), α-linolenic | 2.00 ± 0.61 | 3.21 ± 1.12 | 3.17 ± 1.08 |
| 20:4(ω-4), arachidonic | 0.53 ± 0.38 | 1.07 ± 0.21 | 1.82 ± 0.34 |
| 20:5(ω-3), eicosapentaenoic | - | 0.82 ± 0.28 | 2.51 ± 0.38 |
| 22:6(ω-3), docosahexaenoic | - | 0.74 ± 0.32 | 2.14 ± 0.03 |
| Others | 0.53 ± 0.26 | 0.23 ± 0.12 | 0.36 ± 0.24 |

Data are means ± SD (n=18).

**Table 3.** Sequences of RT-PCR primers for gene expression analysis.

| Target | GenBank accession number | Direction | Sequence (5'→3') |
|---|---|---|---|
| *CD80* | NM_005191.3 | Forward | GGGAAAGTGTACGCCCTGTA |
| | | Reverse | GCTACTTCTGTGCCCACCAT |
| *CD64* | NM_000566.3 | Forward | GTCCAAATCTCCAAGTGCGG |
| | | Reverse | CCCAAGTATGAGAGCAGCGT |
| *MRC1* | NM_138806 | Forward | GGCGGTGACCTCACAAGTAT |
| | | Reverse | ACGAAGCCATTTGGTAAACG |
| *CD200R* | NM_138940.2 | Forward | GTTGCCCTCCTATCGCATTA |
| | | Reverse | TGGAAATTCCCATCAGGTGT |
| *CD209* | NM_021155.3 | Forward | CCAAAGGAGGAGACAAGCAG |
| | | Reverse | GGACGACACGTTCAGTGTGA |
| *CD1a* | NM_001763.2 | Forward | ATATGGCCAAGCATTTCTGC |
| | | Reverse | ATCCTGAGACATGGCACACA |
| *HCA2* | NM_177551.3 | Forward | CACCGACAAGCAGAGAAACA |
| | | Reverse | CACAGCAAAATGGAGCAGAA |
| *PLA2G4D* | NM_178034.3 | Forward | TGGGAAGGAGGTGACTATGG |
| | | Reverse | CTCATCCTCCTGCAGGTCTC |
| *PTGS1* | NM_000962.3 | Forward | GAGTACTGGAAGCCGAGCAC |
| | | Reverse | GCACTCTGGAATGACAAGCA |
| *PTGS2* | NM_000963 | Forward | TTCAAATGAGATTGTGGAAAAAT |
| | | Reverse | AGATCATCTCTGCCTGAGTATCTT |
| *PTGDS* | NM_000954.5 | Forward | AACCAGTGTGAGACCCGAAC |
| | | Reverse | AGGCGGTGAATTTCTCCTTT |
| *PPARG* | NM_005037 | Forward | GCTGTGCAGGAGATCACAGA |
| | | Reverse | GGGCTCCATAAAGTCACCAA |
| *NAMPT* | NM_005746.2 | Forward | GCCAGCAGGGAATTTTGTTA |
| | | Reverse | TGATGTGCTGCTTCCAGTTC |

| | | | |
|---|---|---|---|
| *SIRT1* | NM_001142498.1 | Forward<br>Reverse | GCAGATTAGTAGGCGGCTTG<br>TCTGGCATGTCCCACTATCA |
| *GAPDH* | NM_001289746 | Forward<br>Reverse | CACATGGCCTCCAAGGAGTAAG<br>CCAGCAGTGAGGGTCTCTCT |
| *HPRT* | NM_000194 | Forward<br>Reverse | ACCCCACGAAGTGTTGGATA<br>AAGCAGATGGCCACAGAACT |

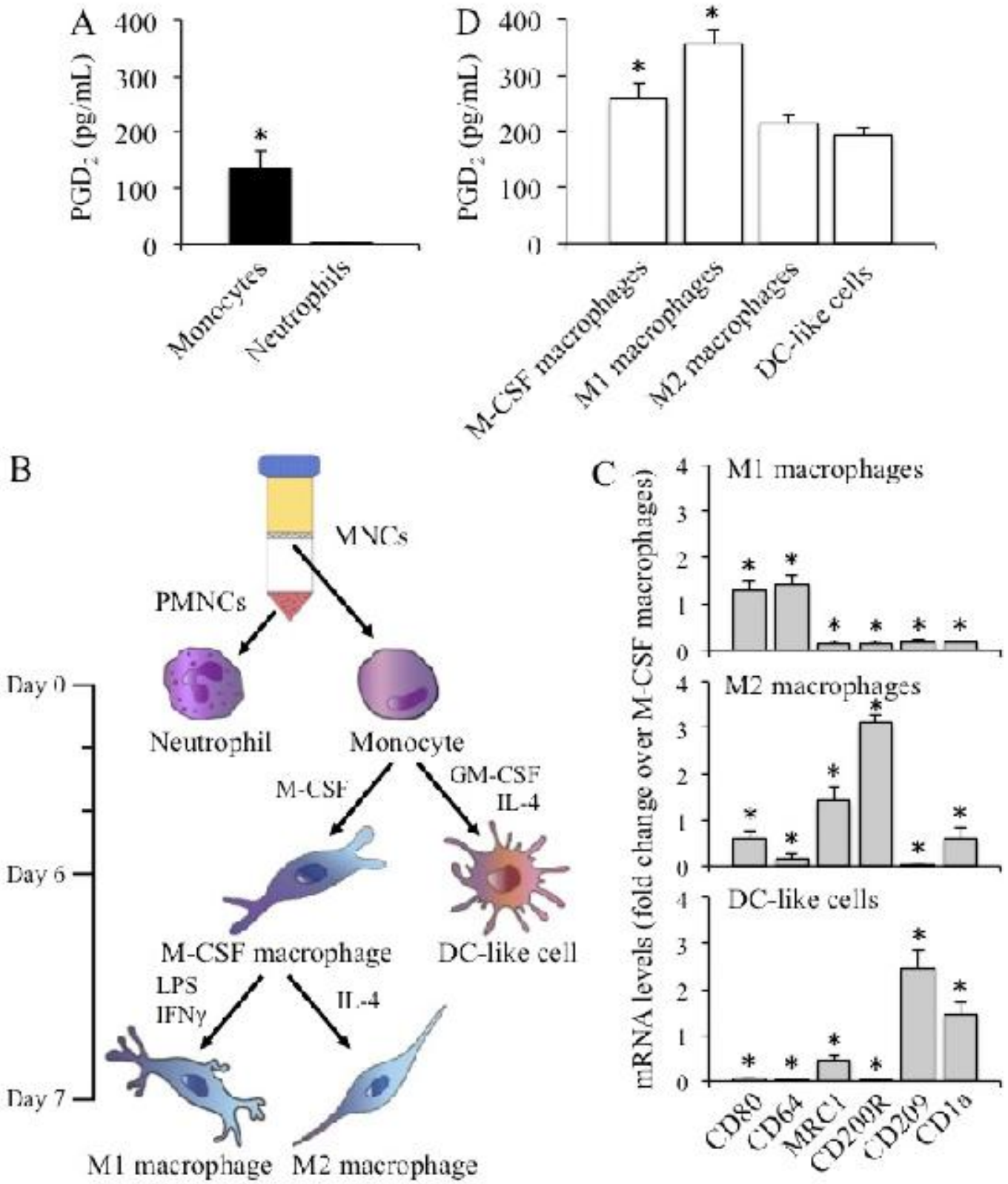
A
400
300
200
100
0
PGD2 (pg/mL)
*
Monocytes
Neutrophils
D
400
300
200
100
0
PGD2 (pg/mL)
*
*
M-CSF macrophages
M1 macrophages
M2 macrophages
DC-like cells
B
MNCs
PMNCs
Neutrophil
Monocyte
Day 0
M-CSF
GM-CSF
IL-4
Day 6
M-CSF macrophage
DC-like cell
LPS
IFNγ
IL-4
Day 7
M1 macrophage
M2 macrophage
C
M1 macrophages
M2 macrophages
DC-like cells
mRNA levels (fold change over M-CSF macrophages)
4
3
2
1
0
CD80
CD64
MRC1
CD200R
CD209
CD1a

Figure 1

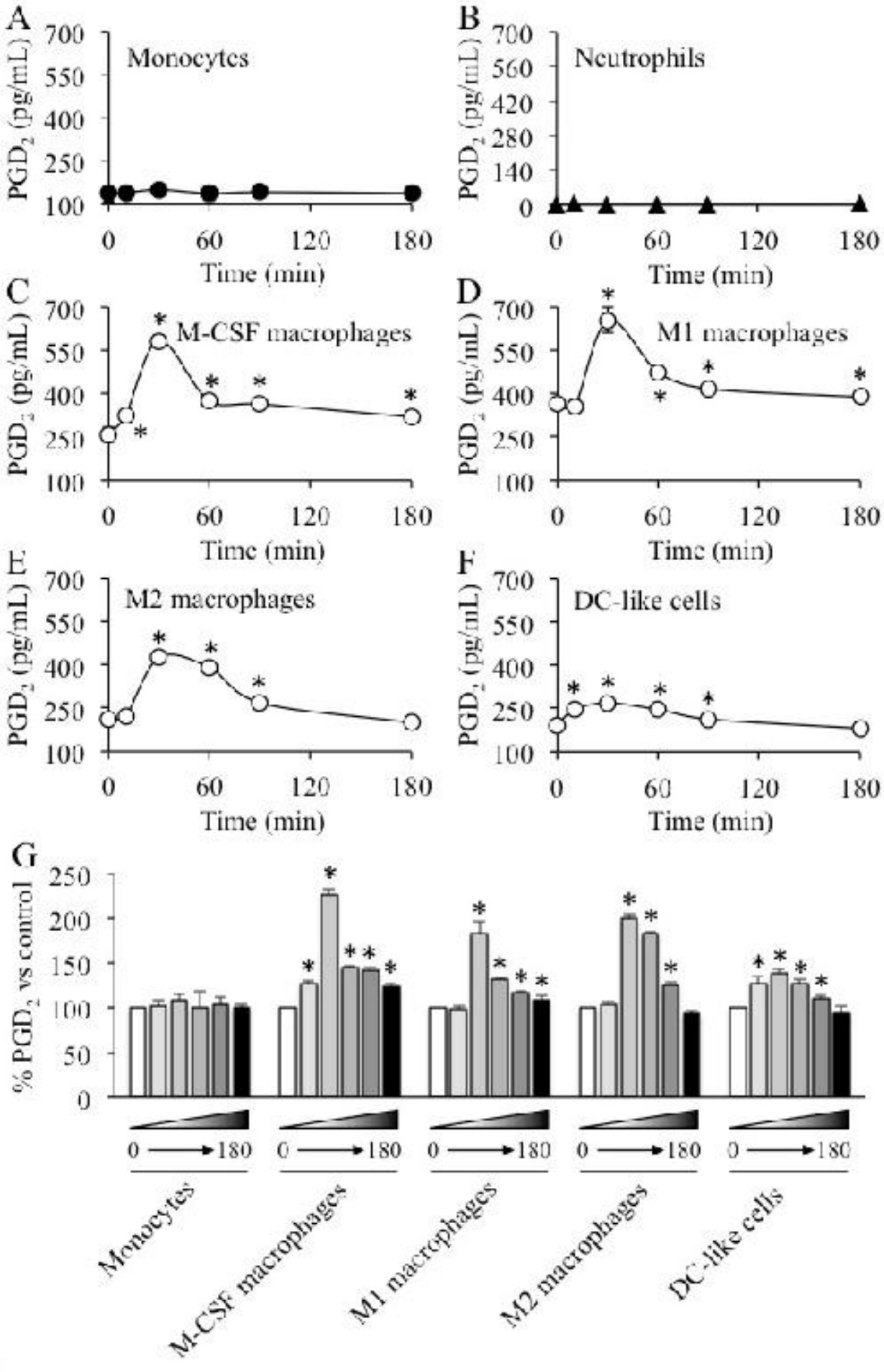
A
Monocytes
PGD2 (pg/mL)
700
550
400
250
100
0
60
120
180
Time (min)
B
Neutrophils
700
560
420
280
140
0
C
M-CSF macrophages
D
M1 macrophages
E
M2 macrophages
F
DC-like cells
G
% PGD2 vs control
250
200
150
100
50
0
0 → 180
Monocytes
M-CSF macrophages
M1 macrophages
M2 macrophages
DC-like cells

Figure 2

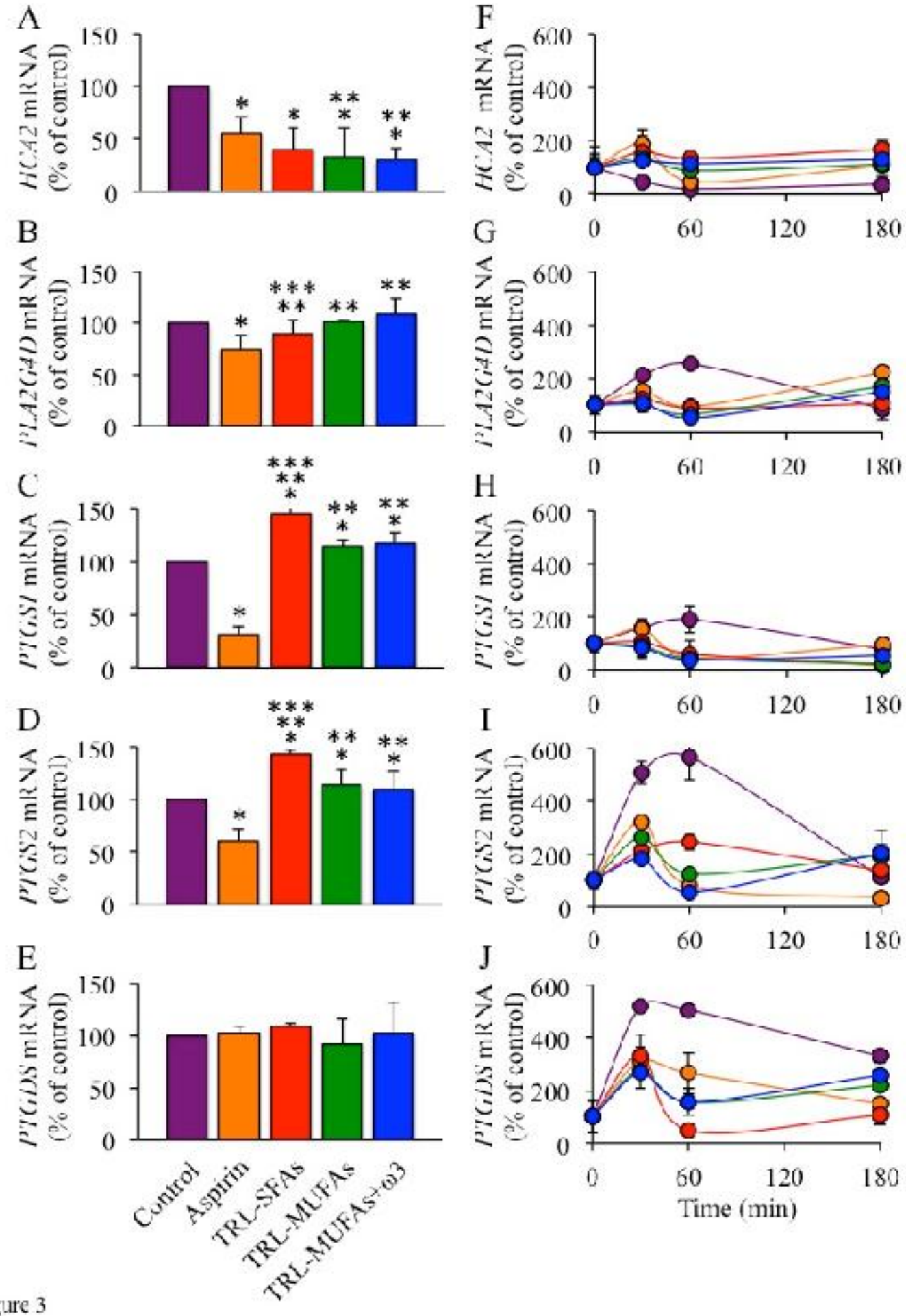
A
B
C
D
E
F
G
H
I
J
HCA2 mRNA (% of control)
PLA2G4D mRNA (% of control)
PTGS1 mRNA (% of control)
PTGS2 mRNA (% of control)
PTGDS mRNA (% of control)
0
50
100
150
200
400
600
0
60
120
180
Time (min)
Control
Aspirin
TRL-SFAs
TRL-MUFAs
TRL-MUFAs+ω3

Figure 3

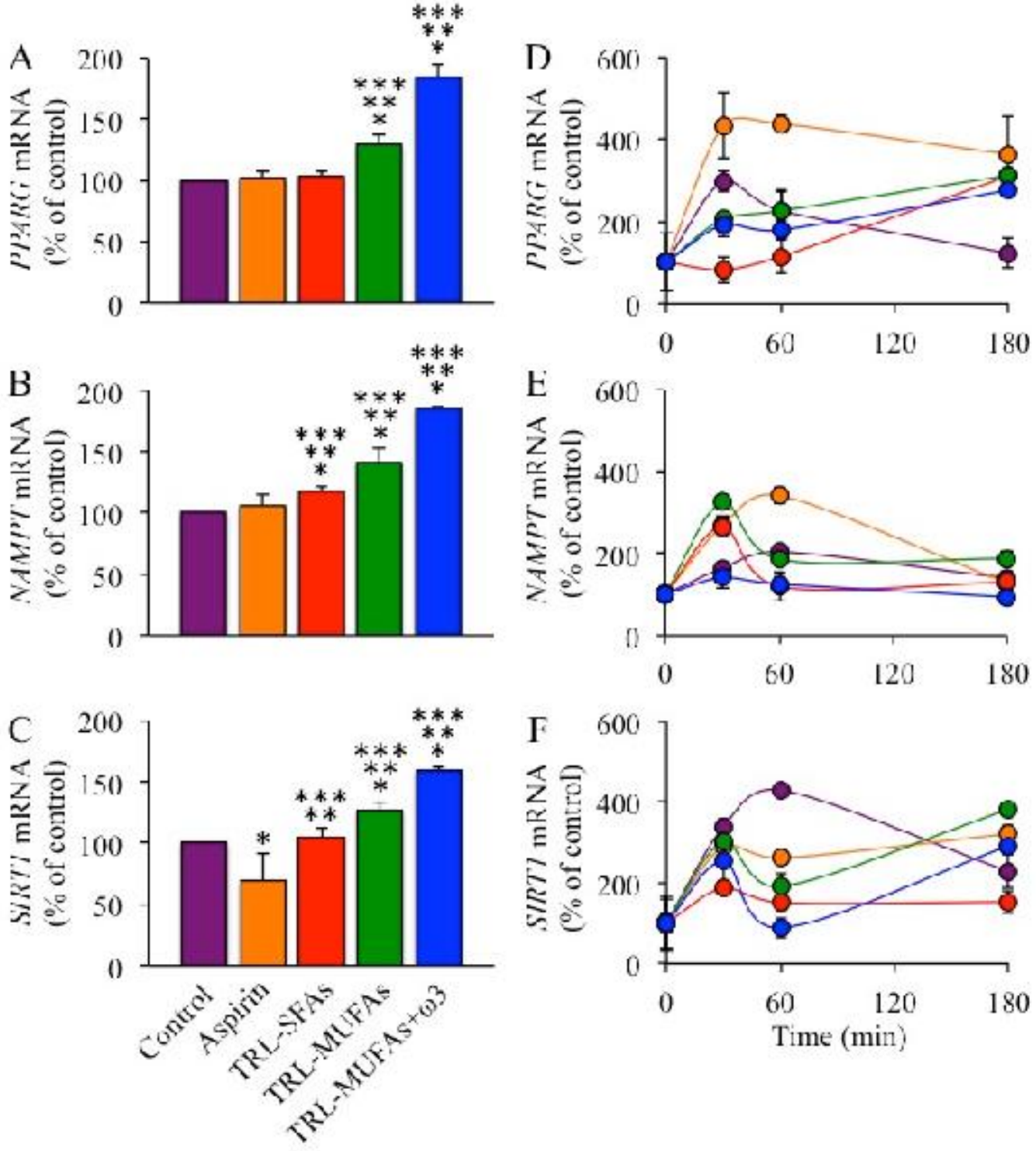
A
PPARG mRNA (% of control)
200
150
100
50
0
B
NAMPT mRNA (% of control)
C
SIRT1 mRNA (% of control)
Control
Aspirin
TRL-SFAs
TRL-MUFAs
TRL-MUFAs+ω3
D
PPARG mRNA (% of control)
600
400
200
0
60
120
180
E
NAMPT mRNA (% of control)
F
SIRT1 mRNA (% of control)
Time (min)

Figure 4

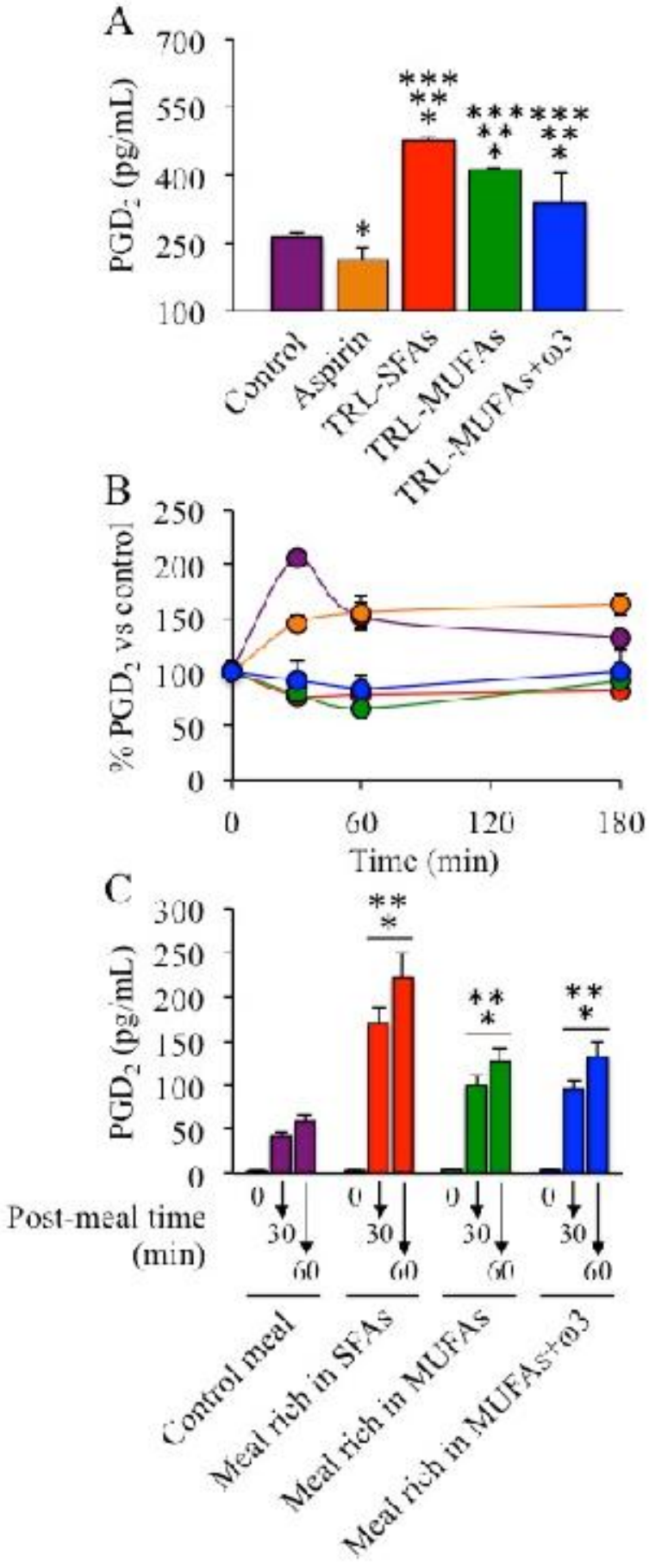
A
PGD2 (pg/mL)
700
550
400
250
100
Control
Aspirin
TRL-SFAs
TRL-MUFAs
TRL-MUFAs+ω3
B
% PGD2 vs control
250
200
150
100
50
0
0
60
120
180
Time (min)
C
PGD2 (pg/mL)
300
250
200
150
100
50
0
Post-meal time (min)
0
30
60
Control meal
Meal rich in SFAs
Meal rich in MUFAs
Meal rich in MUFAs+ω3

Figure 5

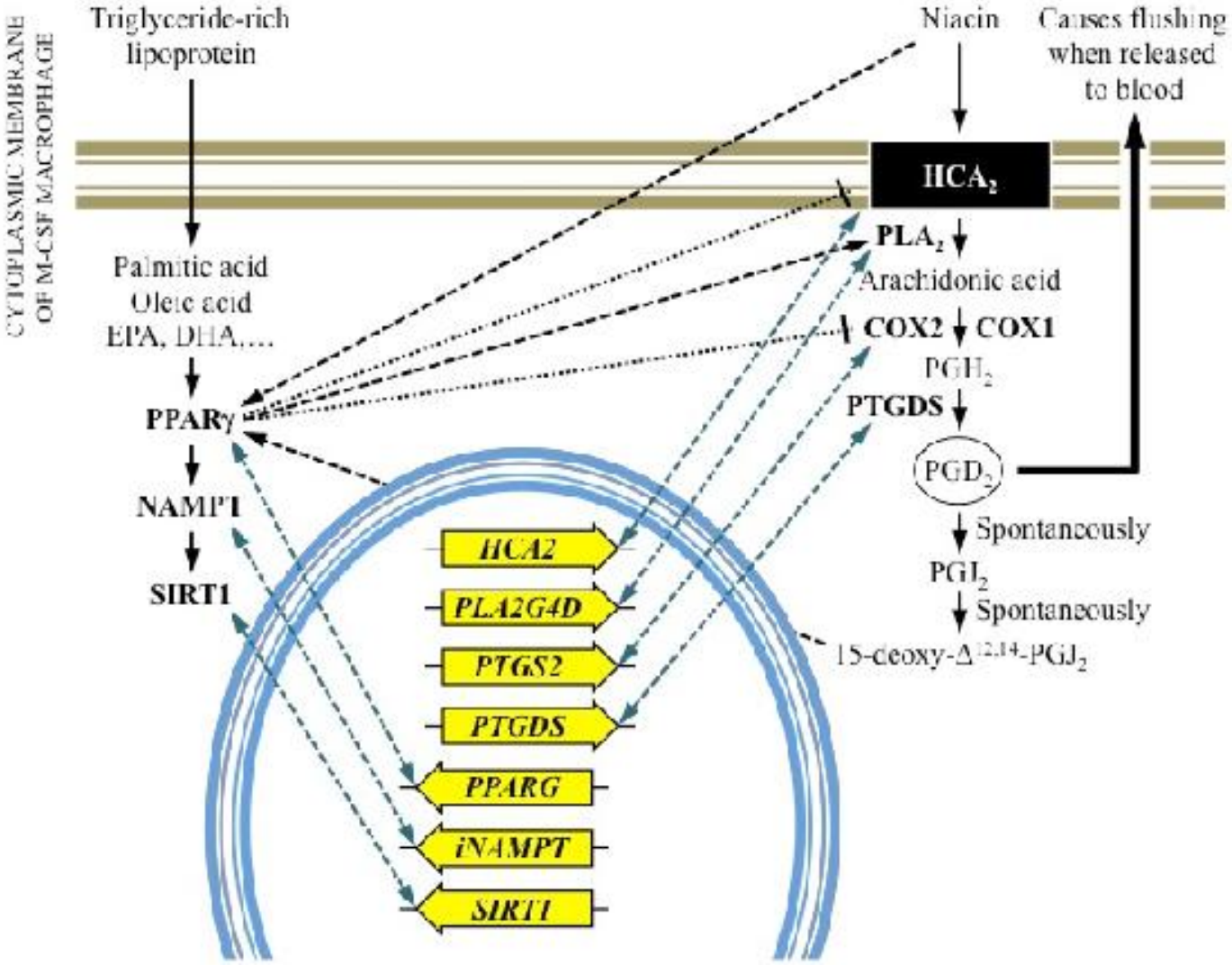
CYTOPLASMIC MEMBRANE OF M-CSF MACROPHAGE
Triglyceride-rich lipoprotein
Palmitic acid
Oleic acid
EPA, DHA,...
PPARγ
NAMPT
SIRT1
Niacin
Causes flushing when released to blood
HCA2
PLA2
Arachidonic acid
COX2
COX1
PGH2
PTGDS
PGD2
Spontaneously
PGI2
Spontaneously
15-deoxy-Δ12,14-PGJ2
HCA2
PLA2G4D
PTGS2
PTGDS
PPARG
iNAMPT
SIRT1

Figure 6