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The effects of exogenous fatty acids and niacin on human monocyte-macrophage plasticity

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Abbreviations

DHA: docosahexaenoic acid, **EPA:** eicosapentaenoic acid, **FA:** fatty acid, **GM-**

CSF: granulocyte-macrophage colony-stimulating factor, **HPRT:** hypoxanthine

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3 101 activated macrophages (also called M2 macrophages) are stimulated by IL-4 or
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5 102 IL-13 and their typical characteristics include high levels of scavenging
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7 103 molecules, high expression of mannose (e.g., CD206) and galactose receptors,
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9 104 CD200R, transforming growth factor β (TGF β), peroxisome proliferator-
10
11 105 activated receptor γ (PPAR γ), high levels of anti-inflammatory cytokines (e.g.,
12
13 106 IL-10), and low levels of pro-inflammatory cytokines [7].
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17 In previous studies, the ingestion of fatty meals has been noticed to
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19 108 induce not only hypertriglyceridemia by virtue of an abrupt intestinal production
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21 109 of triglyceride-rich lipoproteins (TRLs) but also an increase in the plasma levels
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23 110 of cytokines and chemokines that prime the activation of circulating monocytes
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25 111 in humans [8-10]. It has been demonstrated that both monocytes and
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27 112 macrophages are functionally equipped with apoB48 receptor to take up
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29 113 postprandial TRLs, resulting in foam cell formation, which is the first cellular
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31 114 hallmark of the atherosclerotic process [11]. Interestingly, the severity of
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33 115 atherosclerosis is largely influenced by increased leukocyte activation and by
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35 116 long-lasting high circulating postprandial TRLs [12,13]. The fatty acid
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37 117 composition in triglycerides of postprandial TRLs faithfully represents the fatty
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39 118 acid composition of dietary fats ingested. Therefore, the postprandial TRLs
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41 119 constitute an exceptional physiological metabolic entity to explore the relevance
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43 120 of dietary, exogenous fatty acids in the human monocyte-macrophage biology.
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45 121 We previously demonstrated that postprandial TRLs regulate human
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47 122 macrophage-derived foam cells in a fatty acid-dependent manner (SFAs >
48
49 123 MUFAs) [11, 14, 15]. To gain insights into these questions, the present study
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51 124 was aimed to assess whether the type of predominant fatty acids in
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53 125 postprandial TRLs, in particular those enriched in SFAs, MUFAs or MUFAs plus
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ABSTRACT

Scope: Macrophage plasticity allows adapting to different environments, having a dual activity in inflammatory-related diseases. Our hypothesis is that the type of dietary fatty acids into human postprandial triglyceride-rich lipoproteins (TRLs), alone or in combination with niacin (vitamin B3), could modulate the plasticity of monocytes-macrophages.

Methods and results: We isolated TRLs at the postprandial peak from blood samples of healthy volunteers after the ingestion of a meal rich in saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) or MUFAs plus omega-3 long-chain polyunsaturated fatty acids (LCPUFAs). Autologous monocytes isolated at fasting were first induced to differentiate into naïve macrophages. We observed that postprandial TRL-MUFAs, particularly in combination with niacin, enhance competence to monocytes to differentiate and polarise into M2 macrophages. Postprandial TRL-SFAs made polarised macrophages prone to an M1 phenotype. In contrast to dietary SFAs, dietary MUFAs in the meals plus immediate-release niacin primed circulating monocytes for a reduced postprandial pro-inflammatory profile.

Conclusion: Our study underlines a role of postprandial TRLs as a metabolic entity in regulating the plasticity of the monocyte-macrophage lineage and also brings an understanding of the mechanisms by which dietary fatty acids are environmental factors fostering the innate immune responsiveness in humans.

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3 76 **1 Introduction**
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5 77 Vitamin B3 (niacin or nicotinic acid) is a water-soluble vitamin that can be
6
7 78 effective for treatment of chronic high-grade inflammatory diseases such as
8
9 79 atherosclerosis [1]. Many of beneficial effects of niacin are mediated via the G
10
11 80 protein-coupled receptor 109A/hydroxycarboxylic acid 2 receptor
12
13 81 (GPR109A/HCA2), which is highly expressed in adipose tissue and
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15 82 macrophages [2].
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17

18 83 Serving to defend the organism from infection, damaged or activated
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20 84 cells, macrophages are a component of innate immunity with remarkable
21
22 85 versatility of characteristic features that enables them to promote inflammation
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24 86 and to turn the inflammatory response off when it is no longer needed [3]. This
25
26 87 macrophage phenotypic heterogeneity often depends on the microenvironment,
27
28 88 particularly of the surrounding gradients of cytokines and growth factors, such
29
30 89 as granulocyte-macrophage (GM-CSF) and macrophage (M-CSF) colony-
31
32 90 stimulating factors, which are implicated in the differentiation of naïve
33
34 91 macrophages from monocytes [4]. Another level of heterogeneity between
35
36 92 macrophages is related to their activation/polarisation status [5]. As one
37
38 93 extreme of different states of macrophage activation, classically activated
39
40 94 macrophages (also called M1 macrophages) are stimulated by microbial
41
42 95 products such as Toll-like receptor (TLR) ligands such as lipopolysaccharide
43
44 96 (LPS) or pro-inflammatory cytokines such as interferon γ (IFN γ) [6]. These M1
45
46 97 macrophages are characterised by a high expression of pro-inflammatory
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48 98 cytokines (e.g., IL-1 β , TNF- α , and nicotinamide phosphoribosyltransferase,
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50 99 NAMPT), chemokines (e.g., CXCL10), chemokine receptors (e.g., CCR7), and
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52 100 co-stimulatory proteins (e.g., CD80 and TLR4). In contrast, alternatively
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3 101 activated macrophages (also called M2 macrophages) are stimulated by IL-4 or
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5 102 IL-13 and their typical characteristics include high levels of scavenging
6
7 103 molecules, high expression of mannose (e.g., CD206) and galactose receptors,
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9 104 CD200R, transforming growth factor β (TGF β), peroxisome proliferator-
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11 105 activated receptor γ (PPAR γ), high levels of anti-inflammatory cytokines (e.g.,
12
13 106 IL-10), and low levels of pro-inflammatory cytokines [7].
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17 107 In previous studies, the ingestion of fatty meals has been noticed to
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19 108 induce not only hypertriglyceridemia by virtue of an abrupt intestinal production
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21 109 of triglyceride-rich lipoproteins (TRLs) but also an increase in the plasma levels
22
23 110 of cytokines and chemokines that prime the activation of circulating monocytes
24
25 111 in humans [8-10]. It has been demonstrated that both monocytes and
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27 112 macrophages are functionally equipped with apoB48 receptor to take up
28
29 113 postprandial TRLs, resulting in foam cell formation, which is the first cellular
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31 114 hallmark of the atherosclerotic process [11]. Interestingly, the severity of
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33 115 atherosclerosis is largely influenced by increased leukocyte activation and by
34
35 116 long-lasting high circulating postprandial TRLs [12,13]. The fatty acid
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37 117 composition in triglycerides of postprandial TRLs faithfully represents the fatty
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39 118 acid composition of dietary fats ingested. Therefore, the postprandial TRLs
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41 119 constitute an exceptional physiological metabolic entity to explore the relevance
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43 120 of dietary, exogenous fatty acids in the human monocyte-macrophage biology.
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45 121 We previously demonstrated that postprandial TRLs regulate human
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47 122 macrophage-derived foam cells in a fatty acid-dependent manner (SFAs >
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49 123 MUFAs) [11, 14, 15]. To gain insights into these questions, the present study
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51 124 was aimed to assess whether the type of predominant fatty acids in
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53 125 postprandial TRLs, in particular those enriched in SFAs, MUFAs or MUFAs plus
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126 omega-3 long-chain PUFAs (LCPUFAs), alone or in combination with niacin is
127 instrumental in the modulation of human monocyte-macrophage plasticity.

129 **2 Materials and methods**

130 This study was conducted according to Good Clinical Practice Guidelines
131 and in line with the principles outlined in the Helsinki Declaration of the World
132 Medical Association. Informed consent for the study was obtained. This study is
133 part of a clinical trial registered at ClinicalTrials.gov (identifier: NCT02061267).

135 **2.1 TRL isolation, quality, and triglyceride analysis**

136 Oral fat emulsions were prepared according to the method described by
137 the Patent WO/2014/191597 [16] with water, sucrose (30 g/m² of body surface
138 area), fat (50 g/m² of body surface area), emulsifier, and flavouring. Six male
139 volunteers, aged 25 to 35 years, non-smokers, with no medical history of
140 disease known, abnormality of haematological or biochemical parameters were
141 recruited. After an overnight fasting period of 12 h, all of them were given, over
142 three different occasions, an oral fat emulsion containing cow's milk cream
143 (meal rich in SFAs), refined olive oil (meal rich in MUFAs) or refined olive plus a
144 dose of omega-3 LCPUFAs, which consisted of 920 mg of eicosapentaenoic
145 acid (EPA) and 760 mg of docosahexaenoic acid (DHA) (meal rich in
146 MUFAs+omega-3 LCPUFAs). At the postprandial hypertriglyceridemic peak, i.e.
147 2-3 h following the ingestion of the oral fat emulsion, venous blood was
148 collected into K₃EDTA-containing Vacutainer tubes (BD). TRLs were isolated,
149 pooled, and dialysed against cold PBS [17]. TRLs were then immediately stored

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3 150 at -80 °C. The details of quality and triglyceride analysis of TRLs can be found
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5 151 in the Supporting Information.
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7 152

153 2.2 Fat and TRL fatty acid composition

154 The fatty acid composition of cow's milk cream, refined olive oil, and
155 refined olive oil plus omega-3 LCPUFAs was determined, in triplicate from the
156 same lot, by the method described in EEC/796/2002 [18] using a gas
157 chromatography system (Supporting Information Table 1). A similar procedure
158 was followed for fatty acid composition in postprandial TRLs (named TRL-SFAs
159 from cow's milk cream, TRL-MUFAs from refined olive oil, and TRL-PUFAs from
160 refined olive oil plus omega-3 LCPUFAs) (Supporting Information Table 2). The
161 detailed methods can be found in the Supporting Information.
162

163 2.3 Human monocyte isolation

164 The same six volunteers who took part as donors of postprandial TRLs
165 participated as donors of monocytes. After an overnight fasting period of 12 h,
166 peripheral blood samples were drawn from a large antecubital vein and
167 collected into K₃EDTA-containing tubes (BD). Peripheral blood mononuclear
168 cells (PBMCs) were isolated by centrifugation over a Ficoll-Histopaque (Sigma)
169 gradient. Monocytes were isolated from PBMCs using CD14 microbeads and
170 LS columns on a midiMACS system (Miltenyi Biotec). Monocytes were tested
171 for purity by CD14 fluorescein isothiocyanate (FITC) labelling and fluorescence-
172 activated cell sorter (FACS) analysis. The detailed method can be found in the
173 Supporting Information.
174

2.4 Monocyte differentiation and polarisation

Freshly monocytes were induced to differentiate into mature macrophages (M0) by using GM-CSF or M-CSF. M0 macrophages were induced to polarise into M1 or M2 macrophage subtype by long-established cocktail method. GM-M1 and GM-M2 macrophages are referred as those from GM-CSF-derived M0 macrophages. M-M1 and M-M2 macrophages are referred as those from M-CSF-derived M0 macrophages. The detailed methods can be found in the Supporting Information. All of these incubations were done in the absence or presence of autologous postprandial TRLs (100 µg triglycerides/mL) and niacin (100 nM). The experiments were conducted with only once thawed postprandial TRL samples.

2.5 MTT assay

Cell survival was assessed by the MTT colorimetric assay in all of experimental conditions of monocyte differentiation into macrophages and further polarisation in which postprandial TRLs and/or niacin were added. This assay was based on the reduction of dimethylthiazolyldiphenyl-tetrazolium bromide to formazan crystals and was performed as described previously [19].

2.6 RNA extraction and qPCR

Total RNA was extracted from cells using TRIsure™ reagent (Bioline). RNA concentration was assessed in a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). Reverse transcription was performed using 500 ng RNA and iScript™ cDNA Synthesis Kit (Bio-Rad). 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). Ribosomal protein large P0 (*Rplp0*) and hypoxanthine phosphoribosyltransferase (*Hprt*) were used as housekeeping genes. The sequences of the primers used in this study are shown in **Supporting Information Table 3**. Reactions were performed in triplicate and the change in mRNA expression was calculated by using the $2^{-(\Delta\Delta Ct)}$ method. All data were normalised to endogenous reference (*Rplp0* and *Hprt*) gene content and expressed as relative to the control.

2.7 Enzyme-linked immunosorbent assay

Cytokine IL-1 β , TNF- α , IL-10, and chemokine CXCL10 levels were measured in cell culture supernatants by ELISA (Diacclone). Absorbance was read at 450 nm using a Multiskan Spectrum microplate reader (Thermo Labsystems). The quantification was made on the basis of calibration curves prepared for the cytokines and chemokine analysed. Results are expressed as pg/mL of cell culture supernatant.

2.8 Human intervention: acute response to dietary fats and niacin

The same six volunteers who took part as donors of postprandial TRLs or fasting leukocytes participated in this part of the study. After an overnight fasting period of 12 h, all of them were given, over three different occasions, an oral fat emulsion (meal rich in SFAs, meal rich in MUFAs or meal rich in MUFAs plus omega-3 LCPUFAs) as indicated above and a single dose of 2 g immediate-release niacin (Twinlab, UT, USA). The participants also consumed the same test meal without fat (including niacin), as a control meal. Peripheral blood

225 samples were drawn from a large antecubital vein at fasting and at the
226 postprandial hypertriglyceridemic peak, i.e. 2-3 h, and collected into K₃EDTA-
227 containing tubes (BD). Monocytes were then isolated and differentiated into
228 naïve GM-M0 and M-M0 macrophages as described above.

230 **2.9 Statistical analysis**

231 The statistics was performed using the GaphPad Prism v5.0. The
232 experimental results are expressed as the mean ± SD of six independent
233 experiments (one per volunteer) performed in triplicate. Statistical significances
234 were assessed using a one-way analysis of the variance (ANOVA) for multiple
235 comparisons with Tukey's *post hoc* test. For differences between postprandial
236 TRL treatments in combination or not with niacin, we used a two-way ANOVA
237 with Bonferroni's *post hoc* test. A *p*-value of less than 0.05 was considered
238 statistically significant.

240 **3 RESULTS**

241 **3.1 GM-CSF and M-CSF pre-differentiation of human monocytes favours**
242 **an M1 and M2 activation status, respectively, in polarised macrophages**

243 In the context of the ability of GM-CSF and M-CSF to induce human
244 monocyte differentiation into naïve M0 macrophages, we first compared their
245 influence on the gene expression pattern of M1 and M2 markers in polarised
246 macrophages (GM-M1, M-M1, GM-M2, and M-M2). As expected, the mRNA
247 levels of M1 macrophage gene markers (*Cd80*, *Ccr7*, *Il1b*, *Tnfa*, and *Nampt*)
248 were overrepresented in GM-M1 and M-M1 macrophages with respect to GM-
249 M2 and M-M2 macrophages (Supporting Information Figure 1). The mRNA

levels of M2 macrophage gene markers (*Cd200r*, *Cd206*, *Il10*, *Tgfb*, and *Pparg*) were also overrepresented in GM-M2 and M-M2 macrophages with respect to GM-M1 and M-M1 macrophages. However, GM-M1 macrophages had enhanced transcriptional activity of M1 macrophage gene markers (*Cd80*, *Ccr7*, *Il1b*, and *Tnfa*) when compared to M-M1 macrophages, whereas M-M2 macrophages had upregulated *Tgfb* and *Pparg* gene expression when compared to GM-M2 macrophages. Therefore, GM-M1 and M-M2 macrophages and their corresponding naïve GM-M0 and M-M0 macrophages were used for further evaluation of postprandial TRLs and niacin on human monocyte-macrophage plasticity.

3.2 GM-CSF but not M-CSF pre-differentiation of human monocytes increases mitochondrial activity in polarised macrophages. Role of postprandial TRLs and niacin

The mitochondrial activity by using the MTT assay was assessed in the above-mentioned cells. As shown in Figure 1A, GM-M1 macrophages exhibited stimulated mitochondrial activity, remaining comparable the values of GM-M0, M-M0, and M-M2 macrophages. Autologous postprandial TRLs further increased, with no differences among TRL-SFAs, TRL-MUFAs, and TRL-PUFAs, the mitochondrial activity of GM-M1 macrophages (Figure 1B) in a dose-dependent manner with a typical sigmoidal shape (Figure 1C); however, no effects were observed in GM-M0 (Figure 1B), M-M0 (Figure 1D) or M-M2 (Figures 1D and 1E) macrophages. Niacin did no affect the mitochondrial activity of any macrophage subtype (Figure 1F).

275 **3.3 Postprandial TRL-SFAs favours an M1 whereas niacin an M2 activation**
276 **status in polarised macrophages**

277 In gaining deeper insight into the role of autologous postprandial TRLs
278 and niacin on human monocyte differentiation and macrophage polarisation
279 under the influence of GM-CSF and M-CSF, we observed that postprandial
280 TRL-SFAs particularly promoted the transcriptional activity of M1 macrophage
281 gene markers (*Cd80* and *Ccr7*) in GM-M1 macrophages (Figures 2A and 2B). In
282 contrast, niacin not only reduced the mRNA levels of *Cd80* and *Ccr7* genes in
283 GM-M1 macrophages but also promoted the transcriptional activity of M2
284 macrophage gene marker *Cd206*, with no effect on *Cd200r* gene expression, in
285 M-M2 macrophages (Figures 2C and 2D). Postprandial TRLs did not affect the
286 activation status of GM-M0 or M-M0 macrophages. Interestingly, when
287 postprandial TRL-SFAs, TRL-MUFAs or TRL-PUFAs and niacin were added
288 together, niacin abruptly reduced the postprandial-TRL-induced transcriptional
289 activity of M1 macrophage gene markers in GM-M1 macrophages; in contrast,
290 niacin increased the transcriptional activity of *Cd206* gene in M-M0
291 macrophages in the presence of any postprandial TRL and in M-M2
292 macrophages only in the presence of postprandial TRL-MUFAs or TRL-PUFAs.
293 Similar findings were observed for the other M1 (*Il1b*, *Tnfa*, and *Nampt*) and M2
294 (*Il10*, *Tgfb*, and *Pparg*) gene markers (data not shown). Furthermore, the
295 release of pro-inflammatory cytokines IL-1 β (Supporting Information Figure 2A)
296 and TNF- α (Supporting Information Figure 2B) was increased into the medium
297 of GM-M1 and GM-M0 macrophages only in the presence of postprandial TRL-
298 SFAs. The release of pro-inflammatory chemokine CXCL10 was also increased
299 into the medium of GM-M0 macrophages only in the presence of postprandial

TRL-SFAs and into the medium of GM-M1 macrophages in the presence of any postprandial TRL but notably of postprandial TRL-SFAs (Supporting Information Figure 2C). The release of anti-inflammatory cytokine IL-10 was increased into the medium of M-M0 macrophages only in the presence of postprandial TRL-PUFAs and decreased into the medium of M-M2 macrophages in the presence of any postprandial TRL (Supporting Information Figure 2D). The majority of these effects on release of pro- and anti-inflammatory mediators were enhanced when postprandial TRLs and niacin were added together. In M-M2 macrophages, the release of IL-10 was decreased only in the presence of postprandial TRL-SFAs and niacin.

4.4 In contrast to a meal rich in SFAs, meals rich in MUFAs co-ingested with immediate-release niacin favours the priming of postprandial monocytes to naïve GM-M0 and M-M0 macrophages distant from an M1-like phenotype

In establishing the physiological relevance of these findings, naïve GM-M0 and M-M0 macrophages obtained from postprandial monocytes of healthy volunteers subjected to the above-mentioned oral fat emulsions and to a non-fat (control) meal, in combination with a single-dose of immediate-release niacin, were analysed for gene expression of M1 and M2 markers, respectively. Co-ingestion of the SFA meal, but not of MUFA or PUFA meal, and niacin primed naïve GM-M0 macrophages by inducing the upregulation of M1 macrophage gene markers such as *Cd80* (Figure 3A), *CCr7* (Figure 3B), *Tlr4* (Figure 3C), *Tnfa* (Figure 3D), *Cxcl10* (Figure 3E), and *Nampt* (Figure 3F). The SFA meal also induced the downregulation of M2 macrophage gene markers such as

325 *Cd200r* (Figure 4A), *Cd206* (Figure 4B), *Tgfb* (Figure 4C), and *Pparg* (Figure
326 4D) in M-M0 macrophages. In contrast, the co-ingestion of the MUFA or PUFA
327 meal and niacin promoted the downregulation of *Nampt* gene in GM-M0
328 macrophages and the upregulation of *Cd200r* gene in M-M0 macrophages
329 when compared to the control meal and niacin. Furthermore, the MUFA and
330 PUFA meals reduced the transcriptional activity of *Cd2006* and *Tgfb* genes but
331 to a lesser extent than the SFA meal and niacin.

332
333 **5 DISCUSSION**

334 Systemic dyslipidaemias are involved in the aetiology of type-2 diabetes
335 and metabolic syndrome promoting atherosclerosis. The accumulation of
336 monocyte-derived macrophages laden with lipids through the uptake of
337 atherogenic lipoproteins in the vascular intima is the hallmark of plaque
338 development and its complications [20]. It is widely agreed that, compared to
339 diets rich in SFAs or carbohydrates, diets with relatively high MUFA content
340 may elicit benefits against cardiovascular risk outcomes. In the present study,
341 we expand this knowledge and demonstrate that exogenous fatty acids in
342 postprandial TRLs exhibit the capacity of regulating the monocyte-macrophage
343 plasticity.

344 Macrophages are plastic cells whose physiological functions are at least
345 in part dependent of their origin and their microenvironment. The classically
346 activated macrophages (M1 phenotype) are considered as pro-inflammatory,
347 whereas the alternatively activated macrophages (M2 phenotype) are
348 considered as anti-inflammatory and involved in tissue repair [21].

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3 349 We are aware that the M1/M2 nomenclature is an oversimplification and
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5 350 represents the extremes of a continuum of functional phenotypes *in vivo*, which
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7 351 may be considered a limitation. Nevertheless, our study reinforces the notion
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9 352 that GM-CSF and M-CSF influence on the differentiation process from
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11 353 monocytes to macrophages and their polarisation [22-24], but differently.
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13 354 However, other authors did not find significant differences between GM-CSF
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15 355 and M-CSF stimulatory signals for the polarisation of monocytes into M1-
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17 356 macrophages [25]. It is probable that these discrepancies are due to the
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19 357 experimental design, for example, the use of one type of cytokine for monocyte
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21 358 differentiation and then the use of another type of cytokine for macrophage
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23 359 polarisation [26]. To our knowledge, the present study is the first to establish
24
25 360 that GM-CSF and M-CSF do not equally promote human monocyte
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27 361 differentiation into naïve macrophages. Our study also shows that GM-CSF
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29 362 favours naïve macrophages more prone to M1 polarisation, whereas M-CSF to
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31 363 M2 polarisation.

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34 364 Besides distinct functions and gene expression profiles of M1 and M2
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36 365 macrophages, they exhibit contrasted metabolic activities [27]. Metabolism in
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38 366 M1 macrophages is characterised by high levels of glycolysis and flux through
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40 367 the pentose phosphate pathway, and an elevation in certain Krebs cycle
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42 368 intermediates, which occurs in the matrix of the mitochondrion [28]. M1
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44 369 macrophages also have increased synthesis of fatty acids requiring increased
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46 370 energy expenditure and therefore increased mitochondrial activity. Our study
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48 371 confirmed the higher mitochondrial activity in M1 macrophages than in M2
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50 372 macrophages and more importantly that postprandial TRLs promote, probably
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52 373 as a source of fatty acids, the mitochondrial activity in M1 macrophages but not
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3 374 in naïve macrophages or M2-polarised macrophages. Mitochondrial activity has
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5 375 been implicated as a cause of aging, and mitochondrial dysfunction and
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7 376 reactive oxygen species production have been linked to neurodegeneration,
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9 377 cancer, and autoimmunity [29]. Noteworthy, niacin did not affect the
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11 378 mitochondrial activity in our model cells but buffered the postprandial-TRL-
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13 379 induced mitochondrial activity in M1 macrophages, unveiling novel beneficial
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16 380 effects of niacin in regulating metabolic functions of selective macrophage
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19 381 subtypes.

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21 382 Gene expression markers for GM-M1 and M-M2 macrophages were
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23 383 analysed in the absence or presence of autologous postprandial TRLs and
24
25 384 niacin. Our data showed that postprandial TRL-SFAs skewed naïve
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27 385 macrophages toward M1-like macrophages, whereas M2 macrophage gene
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29 386 markers were compromised, contrarily to that observed with postprandial TRL-
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31 387 MUFAs and TRL-PUFAs, skewing naïve macrophages toward M2-like
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33 388 macrophages. These findings are in accordance with previous studies in which
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35 389 SFAs, via TRL2, TLR4, and NF- κ B activation, were shown to be inducers of
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37 390 M1 macrophages, an effect that was inhibited by MUFAs and PUFAs [30-34]. In
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39 391 our study, we provide fatty acids in a more physiological way by using
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42 392 postprandial TRLs. It was interesting to observe that niacin had an inhibitory
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44 393 role to modulate the postprandial-TRL-induced macrophage polarisation into M1
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46 394 macrophages and had an activator role to modulate the macrophage
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48 395 polarisation into M2 macrophages when co-incubated with postprandial TRLs
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50
51 396 rich in MUFAs. These findings were consistent with the release of pro-
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53 397 inflammatory and anti-inflammatory cytokines and demonstrate that the type of
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56 398 predominant fatty acids in the diet could have a critical role in the plasticity of
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human macrophages *in vitro*. Dietary SFAs acutely induced pro-inflammatory macrophages, whereas niacin and dietary MUFAs and omega-3 LCPUFAs favoured the other extreme of a dynamic changing state of macrophages at different levels of activation represented by anti-inflammatory macrophages. In addition, we analysed gene expression of M1 and M2 markers in naïve M-M0 macrophages obtained from postprandial monocytes of healthy volunteers subjected to SFA, MUFA, and PUFA meals in combination with a single-dose of immediate-release niacin. Immediate-release niacin is usually completely absorbed within 1 to 2 hours, whereas dietary fatty acids are largely incorporated into postprandial TRLs and released into the blood stream with a peak occurring 2 to 3 hours after fatty food consumption. Therefore, when co-ingested together, postprandial TRLs and niacin may coexist with circulating monocytes prior to their migration to vascular endothelium and differentiation to macrophages. Furthermore, postprandial TRL are irreversibly trapped (in contrast to LDL, which are reversibly trapped) in the subendothelial space [35] where they, and niacin, may interact with surrounding cells, including macrophages [36]. In support of this notion, the human monocyte-macrophage lineage is well equipped with genes encoding receptors that recognize postprandial TRLs [11, 36] and niacin [2, 37]. Also relevant, a regulatory crosstalk and interference between predominant fatty acids from postprandial TRLs and niacin pathways have been recently reported in human myeloid cells [38]. The mechanistic basis of these effects remains unclear. In the present study, we have further established that high-fat meals primed the postprandial monocytes in healthy volunteers to differentiate into naïve macrophages with predisposition to polarise into M1-like macrophages (SFA meal) or into M2-like

424 macrophages (MUFA meal, PUFA meal) in the setting of immediate-release
425 niacin treatment, which measure the physiological relevance of our *in vitro*
426 findings on the role of dietary fatty acids in postprandial TRLs in the plasticity of
427 human monocyte-macrophage axis.

429 **Conflicts of interest**

430 The authors state no conflict of interest.

432 **Author contributions**

433 S.M., R.A., F.J.G.M., and S.L. designed the study. D.R., M.P.C., and M.C.N.
434 performed *in vitro* studies. S.M., B.B., and S.L. performed human intervention.
435 S.M., F.J.G.M., and S.L. interpreted the data and wrote the paper. All authors
436 discussed the results and implications and commented on the manuscript at all
437 stages.

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569 **FIGURE LEGENDS**

570 **Figure 1.**

571 Mitochondrial activity (A) and its influence by postprandial TRLs and niacin (NA)
572 in human GM-M0, GM-M1, M-M0, and M-M2 macrophages. (B) GM-M0 and
573 GM-M1 macrophages in the presence or absence of postprandial TRLs; (C)
574 GM-M1 macrophages, time-course in the presence or absence of postprandial
575 TRLs and niacin; (D) M-M0 and M-M2 macrophages in the presence or
576 absence of postprandial TRLs; (E) M-M2 macrophages, time-course in the
577 presence or absence of postprandial TRLs and niacin; (F) GM-M1 and M-M2 in
578 the absence or presence of niacin. Freshly human monocytes from six healthy
579 male volunteers were differentiated with GM-CSF (50 ng/mL) or M-CSF (50
580 ng/mL) and then polarised with LPS (100 ng/mL) plus IFN γ (20 ng/mL) or IL-4
581 (20 ng/mL). By using GM-CSF or M-CSF, GM-M0 and GM-M1 or M-M0 and M-
582 M2 macrophages were obtained. Cells were incubated with or without (control)
583 autologous postprandial TRLs (100 μ g triglycerides/mL) and NA (100 nM).
584 Postprandial TRLs were obtained from the above volunteers after the ingestion
585 of oral fat emulsions (cow's milk cream: TRL-SFAs; refined olive oil: TRL-
586 MUFAs; refined olive oil plus omega-3 LCPUFAs: TRL-PUFAs). Mitochondrial
587 activity was determined by MTT assay. Values are expressed as the mean $\pm$
588 SD of six independent experiments (one per volunteer) performed in triplicate.
589 Data with different lowercase letters are significantly different at $p<0.05$
590 according to the one-way analysis of variance statistical analysis followed by
591 Tukey *post hoc* test. Two-way ANOVA followed by a Bonferroni *post hoc* test
592 was used when appropriate. # Significantly different with respect to NA treated
593 cells.

594

595 **Figure 2.**

596 Expression of M1 and M2 gene markers in human GM-M0, GM-M1, M-M0, and
597 M-M2 macrophages in the presence or absence of postprandial TRLs and
598 niacin (NA). M1 gene markers: (A) *Cd80*; (B), *Ccr7*. M2 gene markers: (C)
599 *Cd200r*, (D) *Cd206*. Freshly human monocytes from six healthy male volunteers
600 were differentiated with GM-CSF (50 ng/mL) or M-CSF (50 ng/mL) and then
601 polarised with LPS (100 ng/mL) plus IFN γ (20 ng/mL) or IL-4 (20 ng/mL). By
602 using GM-CSF or M-CSF, GM-M0 and GM-M1 or M-M0 and M-M2
603 macrophages were obtained. Cells were incubated with or without (control)
604 autologous postprandial TRLs (100 μ g triglycerides/mL) and NA (100 nM).
605 Postprandial TRLs were obtained from the above volunteers after the ingestion
606 of oral fat emulsions (cow's milk cream: TRL-SFAs; refined olive oil: TRL-
607 MUFAs; refined olive oil plus omega-3 LCPUFAs: TRL-PUFAs). Cells without
608 TRL and NA treatment were used as control cells for data
609 normalization/analysis. Values are expressed as the mean $\pm$ SD of six
610 independent experiments (one per volunteer) performed in triplicate. Data with
611 different lowercase (GM-M0 and M-M0 macrophages) and uppercase (GM-M1
612 and M-M2 macrophages) letters are significantly different at $p < 0.05$ according
613 to the two-way analysis of variance statistical analysis followed by Bonferroni
614 *post hoc* test.

615

616 **Figure 3.**

617 Expression of M1 gene markers in GM-M0 macrophages from human
618 postprandial monocytes after the co-ingestion of oral fat emulsions and a single-

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2
3 619 dose of immediate-release niacin (NA). (A) *Cd80*; (B), *Ccr7*; (C) *Tlr4*; (D) *Tnfa*;
4
5 620 (E) *Cxcl10*; (F) *Nampt*. Freshly human postprandial monocytes from six healthy
6
7 621 male volunteers were isolated after the ingestion of oral fat emulsions rich in
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9 622 cow's milk cream (SFA meal), refined olive oil (MUFA meal), refined olive oil
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11 623 plus omega-3 LCPUFAs (PUFA meal), or a non-fat (control) meal in
12
13 624 combination with a single-dose of immediate-release NA. Cells were then
14
15 625 differentiated with GM-CSF (50 ng/mL) into GM-M0 macrophages. Values are
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17 626 expressed as the mean $\pm$ SD of six independent experiments (one per
18
19 627 volunteer) performed in triplicate. Data with different lowercase letters are
20
21 628 significantly different at $p < 0.05$ according to the one-way analysis of variance
22
23 629 statistical analysis followed by Tukey *post hoc* test.
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30 **Figure 4.**

31
32 632 Expression of M2 gene markers in M-M0 macrophages from human
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34 633 postprandial monocytes after the co-ingestion of oral fat emulsions and a single-
35
36 634 dose of immediate-release niacin (NA). (A) *Cd200r*; (B), *Cd206*; (C) *Tgfb*; (D)
37
38 635 *Pparg*. Freshly human postprandial monocytes from six healthy male volunteers
39
40 636 were isolated after the ingestion of oral fat emulsions rich in cow's milk cream
41
42 637 (SFA meal), refined olive oil (MUFA meal), refined olive oil plus omega-3
43
44 638 LCPUFAs (PUFA meal), or a non-fat (control) meal in combination with a single-
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46 639 dose of immediate-release NA. Cells were then differentiated with M-CSF (50
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48 640 ng/mL) into M-M0 macrophages. Values are expressed as the mean $\pm$ SD of six
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50 641 independent experiments (one per volunteer) performed in triplicate. Data with
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52 642 different lowercase letters are significantly different at $p < 0.05$ according to the
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one-way analysis of variance statistical analysis followed by Tukey *post hoc*
test.

For Peer Review

SUPPORTING INFORMATION

2 Materials and methods

2.1 Postprandial TRL isolation, quality, and triglyceride analysis

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). LPS contamination was always <0.2 EU/mL. Triglyceride concentration in postprandial TRLs was determined by colorimetric assay kit TG GPO-POD (Bioscience Medical).

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 LCPUFAs was determined by using a HP-5890 gas chromatograph (Hewlett-Packard) equipped with flame ionization detector and a SP-2380 capillary column (Supelco, 30 m × 0.32 mm) packed with cyanopropyl siloxane (0.25 µm) (Supporting Information Table 1). The initial column temperature was 165 °C, which was held for 10 min, then programmed from 165 °C to 200 °C at 1.5 °C/min. Injector and detector temperature were 250 °C, with the carrier gas H₂. For fatty acid composition in postprandial TRLs (named TRL-SFAs from cow's milk cream, TRL-MUFAs from refined olive oil, and TRL-PUFAs from refined olive oil plus omega-3 LCPUFAs), aliquots of 100 µL were lyophilized. A solution composed 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 N₂ gas. The resulting extract was dissolved in heptane and the fatty acid methyl esters were

694 analysed into a gas chromatography system as described above (Supporting
695 Information Table 2).

697 **2.3 Human monocyte isolation**

698 Briefly, 5×10^5 cells were stained with FITC-conjugated CD14 (BD), and 5×10^5
699 cells were stained with FITC-conjugated mouse IgG2a as a negative isotype control.
700 Cells were incubated in PBS, 0.1% sodium azide, and 20 μ L of the conjugated
701 antibody at room temperature for 15 min, washed, and resuspended in 300 μ L PBS. A
702 FACScanto II flow cytometer and FACSDiva software (BD) were used for the analysis.
703 Purity above 98% was considered acceptable. The monocytes were seeded at a
704 density of 5×10^5 cells/mL and cultured in ultra low attachment flasks in RPMI 1640
705 medium supplemented with L-glutamine, penicillin, streptomycin, and 10% heat-
706 inactivated FBS (complete culture medium).

708 **2.4 Monocyte differentiation and polarisation**

709 Monocytes (5×10^5 cells/mL) were differentiated into naïve GM-M0 and M-M0
710 macrophages by exposure for 6 days to 50 ng/mL GM-CSF and 50 ng/mL M-CSF,
711 respectively. Complete culture medium was replaced every 2 days with fresh medium
712 and the cytokine. GM-M0 and M-M0 macrophages were exposed for additional 24 h to
713 LPS (100 ng/mL) plus IFN γ (20 ng/mL) for M1 polarisation (GM-M1 and M-M1) and to
714 IL-4 (20 ng/mL) for M2 polarisation (GM-M2 and M-M2).

Supporting Information Table 1. Fatty acid composition of dietary fats.

	Cow's milk cream	Refined olive oil	Refined olive oil + omega-3 LCPUFAs
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(n-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(n-9), oleic	25.3 ± 0.71	61.9 ± 1.23	61.5 ± 0.97
18:2(n-6), linoleic	4.27 ± 0.82	7.97 ± 0.65	8.04 ± 0.53
18:3(n-3), α-linolenic	0.39 ± 0.05	1.04 ± 0.13	0.94 ± 0.03
20:5(n-3), eicosapentaenoic	-	-	0.92 ± 0.09
22:6(n-3), docosahexaenoic	-	-	0.72 ± 0.10
Others	0.96 ± 0.42	2.05 ± 1.08	2.01 ± 0.88

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723 Values are expressed as the mean ± SD, n = 3.

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Supporting Information Table 2. Fatty acid composition of postprandial triglyceride-rich lipoproteins (TRLs) from healthy volunteers after the ingestion of oral emulsions rich in cow's milk cream (TRL-SFAs), refined olive oil (TRL-MUFAs), or refined olive oil plus omega-3 LCPUFAs (TRL-PUFAs).

	TRL-SFAs	TRL-MUFAs	TRL-PUFAs
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(n-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(n-9), oleic	22.8 ± 2.03	66.4 ± 3.27	60.7 ± 2.18
18:2(n-6), linoleic	4.24 ± 1.06	8.93 ± 1.27	10.1 ± 1.44
18:3(n-3), α -linolenic	2.00 ± 0.61	3.21 ± 1.12	3.17 ± 1.08
20:4(n-4), arachidonic	0.53 ± 0.38	1.07 ± 0.21	1.82 ± 0.34
20:5(n-3), eicosapentaenoic	-	0.82 ± 0.28	2.51 ± 0.38
22:6(n-3), docosahexaenoic	-	0.74 ± 0.32	2.14 ± 0.03
Others	0.53 ± 0.26	0.23 ± 0.12	0.36 ± 0.24

Values are expressed as the mean ± SD, $n = 6$.

746 **Supporting Information Table 3.** Sequences of RT-PCR primers for gene expression
747 analysis.

Target	GenBank accession number	Direction	Sequence (5'→3')
<i>Cd80</i>	NM_005191.3	Forward	GGGAAAGTGTACGCCCTGTA
		Reverse	GCTACTTCTGTGCCACCAT
<i>Ccr7</i>	NM_001301714	Forward	TCATGGTCTTGAGCCTCTTGA
		Reverse	ATTGTTTCGTGGGCCTACTG
<i>Il1b</i>	NM_000576	Forward	GGGCCTCAAGGAAAAGAATC
		Reverse	TTCTGCTTGAGAGGTGCTGA
<i>Tnfa</i>	NM_000594	Forward	TCCTTCAGACACCCTCAACC
		Reverse	AGGCCCCAGTTTGAATTCTT
<i>Nampt</i>	NM_005746	Forward	AATGTTCTCTTCACGGTGGAAAA
		Reverse	ACTGTGATTGGATACCAGGACT
<i>Cd206</i>	NM_002438	Forward	GGCGGTGACCTCACAAGTAT
		Reverse	ACGAAGCCATTTGGTAAACG
<i>Cd200r</i>	NM_138806	Forward	TGAAGGAAGGGGCTAGAAGGA
		Reverse	AGGTTAGCAGTTCTCCAAGGG
<i>Il10</i>	NM_000572	Forward	ATGAGCATTGAGACTGGGTAAAC
		Reverse	TTTGTAGGGGCTAAGAAACGCAT
<i>Tgfb</i>	NM_000660	Forward	CAATTCCTGGCGATACCTCAG
		Reverse	GCACAACCTCCGGTGACATCAA
<i>Pparg</i>	NM_005037	Forward	GCTGTGCAGGAGATCACAGA
		Reverse	GGGCTCCATAAAGTCACCAA
<i>Cxcl10</i>	NM_001565	Forward	CAAAATTGGCTTGCAGGAAT
		Reverse	AGGAACCTCCAGTCTCAGCA
<i>Tlr4</i>	NM_003257	Forward	CTGCCACATGTCAGGCCTTAT
		Reverse	AATGCCACCTGGAAGACTCT
<i>Rplp0</i>	NM_001002	Forward	TCGACAATGGCAGCATCTAC
		Reverse	ATCCGTCTCCACAGACAAGG
<i>Hprt</i>	NM_000194	Forward	ACCCACGAAGTGTTGGATA
		Reverse	AAGCAGATGGCCACAGAACT

748

749 **Supporting Information Figure 1.**

750 **Expression of M1 and M2 gene markers in human GM-M1, GM-M2, M-M1, and M-M2**

751 **macrophages. M1 gene markers: (A) *Cd80*; (B) *Ccr7*; (C) *Il1b*; (D) *Tnfa*; (E) *Nampt*. M2**

gene markers: (F) *Cd200r*; (G) *Cd206*; (H) *Il10*; (I) *Tgfb*; (J) *Pparg*. Freshly human monocytes from six healthy male volunteers were differentiated with GM-CSF (50 ng/mL) or M-CSF (50 ng/mL) and then polarised with LPS (100 ng/mL) plus IFN γ (20 ng/mL) or IL-4 (20 ng/mL). By using GM-CSF or M-CSF, GM-M0, GM-M1, and GM-M2 or M-M0, M-M1, and M-M2 macrophages were obtained. M-M0 and GM-M0 macrophages were used as control cells for data normalization/analysis. Values are expressed as the mean $\pm$ SD of six independent experiments (one per volunteer) performed in triplicate. Data with different lowercase letters are significantly different at $p < 0.05$ according to the one-way analysis of variance statistical analysis followed by Tukey *post hoc* test.

Supporting Information Figure 2.

Release of pro-inflammatory cytokines and chemokine in human GM-M0 and GM-M1

macrophages, and of anti-inflammatory cytokines in human M-M0 and M-M2

macrophages in the presence or absence of postprandial TRLs and niacin (NA). Pro-

inflammatory cytokines: (A) IL-1 β ; (B), TNF- α . Pro-inflammatory chemokine: (C) CXCL10. Anti-inflammatory cytokines: (D) IL-10. Freshly human monocytes from six healthy male volunteers were differentiated with GM-CSF (50 ng/mL) or M-CSF (50 ng/mL) and then polarised with LPS (100 ng/mL) plus IFN γ (20 ng/mL) or IL-4 (20 ng/mL). By using GM-CSF or M-CSF, GM-M0 and GM-M1 or M-M0 and M-M2 macrophages were obtained. Cells were incubated with or without (control) autologous postprandial TRLs (100 μ g triglycerides/mL) and NA (100 nM). Postprandial TRLs were obtained from the above volunteers after the ingestion of oral fat emulsions (cow's milk cream: TRL-SFAs; refined olive oil: TRL-MUFAs; refined olive oil plus omega-3 LCPUFAs: TRL-PUFAs). Values are expressed as the mean $\pm$ SD of six independent experiments (one per volunteer) performed in triplicate. Data with different lowercase letters are significantly different at $p < 0.05$ according to the one-way analysis of variance statistical analysis followed by Tukey *post hoc* test.

Figure 1

Figure 2

Figure 3

Figure 4