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Inflammatory activation of Macrophages by Triglyceride-Rich Lipoproteins in Atherosclerosis

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Study track: Cell and Systems Physiology

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ABSTRACT

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Background: Inflammation is a key factor in atherosclerotic cardiovascular disease (ASCVD) and is present at all phases. It has been shown that reducing inflammation by blocking cytokine pathways diminishes the risk of stroke and myocardial infarction. Despite the well-established linked between lipoproteins and atherosclerosis, little is known on the specific effect of lipids on inflammation. In this study, we investigated the impact of triglycerides-rich lipoproteins' (TRLs) lipids on inflammation in the context of atherosclerosis.

Methods: TRLs were isolated and purified from pooled plasma of healthy volunteers, and emulsion particles (EPs) generated by sonication using lipids extracted from TRLs. TRLs and EPs were characterized in size, triglycerides, and cholesterol content. THP-1 cells were treated with EPs, TRLs, and modified EPs (oxidation, vortexed, and lipolysis with PLA2), and the release of pro-inflammatory cytokines (IL-1β and TNF-α) was detected with ELISA.

Results: EPs were successfully synthesized by sonication using an ultrasonic probe. EPs induced cytokine secretion from THP-1 cells (N=4). Modified EPs (Oxidized EPs, vortexed EPs, and PLA2-treated EPs) did not increase cytokine secretion (N=4).

Conclusion: Our findings suggest that TRLs' lipids contribute to inflammation and that TRLs may play a crucial role in the pathogenesis and pathophysiology of ASCVD. Inflammatory properties of TRLs should be extensively investigated in the future for the development of preventive and curative strategies.

ABBREVIATIONS

ASCVD	atherosclerosis cardiovascular disease
ApoB	apolipoprotein B
BCA	bicinchoninic acid
CVD	cardiovascular disease
EDTA	ethylenediaminetetraacetic acid
EP	emulsion particle
FFA	free fatty acid
HDL	high-density lipoprotein
IDL	intermediate-density lipoprotein
IL	interleukin
KBr	potassium bromide
LDL	low-density lipoprotein
LPL	lipoprotein lipase
NEFA	non-esterified fatty acid
Ox	oxidized
PBS	phosphate-buffered saline
PCSK9	proprotein convertase subtilisin/kexin type 9
PLA2	phospholipase A2
TBARS	thiobarbituric acid reactive substance assay
TG	triglyceride
TNF-α	tumor necrosis factor alpha
TRL	triglyceride-rich lipoprotein
VLDL	very low-density lipoprotein
Vx	vortexed

AIMS OF THE STUDY

The overall aim of this thesis project was to investigate the impact of TRLs on inflammation in the context of atherosclerosis.

Specifically, the following three aims were addressed:

- Development of a method for the synthesis of emulsion particles (EPs) generated from human TRLs (Chylomicrons, VLDLs, IDLs).
- Investigation of the release of specific markers of inflammation (i.e., IL-1β and TNF-α) by macrophages treated with TRLs.
- Assessment of the effect of lipids on inflammation specifically by treating macrophages with EPs and modified EPs.

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1 INTRODUCTION

1.1 Atherosclerotic cardiovascular disease

Atherosclerosis is the most common form of cardiovascular disease (CVD) and one of the main causes of coronary heart disease. Especially in the west, atherosclerotic CVD (ACVD) is the leading cause of death worldwide (WHO, 2020). It is characterized by an altered lipoproteins metabolism, lipid accumulation in the arteries, and inflammation[1–3]. Atherogenesis can start as early as in childhood and evolves progressively during life, where the formation of fatty plaques in the arterial wall causes the thickening and hardening of the affected artery. Over the age of 40, there is around 50% chance that individuals in general good health develop the condition, and the risk continues to increase with age[4]. Interestingly, ASCVD has been recently linked to premature biological aging and gut microbiome alterations[3], but its etiology and pathophysiology are still not fully understood. The accurate detection and effective treatment of unstable plaques remain difficult, and the usual clinical intervention consists in serum-lipid lowering, achieved by dietary means or medication, as indicated in the 2018 ACC/AHA guideline that emphasizes reducing the risk of ASCVD through lipid management[5].

Lipid-lowering drug therapies are indicated for the primary and secondary prevention of CV events. Primary prevention includes patients with risk factors but without history of CV event. Secondary prevention includes patients with a history of CV events. Lipoprotein-lowering agents include statins (first line), ezetimibe, fibrates, nicotinic acid, bile acid sequestrants, and PCSK9 inhibitors (new group of drugs)[5]. These medications have been proven efficient when combined with lifelong commitment lifestyle modifications.

Although atherosclerosis can develop in any artery, carotid arteries are the most affected, followed by the peripheral arteries and the heart. The coronary arteries are a preferential location for disease initiation, given that branch bifurcation regions are atheroprone anatomical sites[6]. In addition, the composition of the extracellular matrix of the subendothelial space at the coronary arteries promotes the deposition and retention of monocytes and lipoproteins. The anatomical curvature, vessel thickness, extracellular matrix quality, and high flow rate make the heart the predominant location for the build-up of fatty deposits[7,8].

1.2 Atherosclerotic lesion

Atherogenesis is initiated when circulating apolipoprotein B (ApoB)-containing lipoproteins enter the arterial wall. Apo-B containing lipoproteins include all lipoproteins except high-density lipoproteins (HDL). The artery wall is made of three layers: the tunica adventitia, the tunica media, and the tunica intima. The endothelium consists of a layer of endothelial cells on the luminal side of the blood vessel and is directly in contact with blood. It is the most external part of the tunica intima. The tunica intima consists of the endothelium, connective tissue, and a basal layer of elastic tissue also called internal elastic lamina that sits between the tunica intima and the tunica media[9].

Endothelial cells are connected by adherens, tight and gap junctions, forming a highly dynamic and controlled structure, essential for the maintenance of vascular homeostasis. Local and systemic stimuli can change the structure and function of the endothelium, e.g., poor dietary and lifestyle habits negatively affect its integrity. An activated endothelium can display an altered permeability. As a consequence, circulating lipoproteins can come in contact with the luminal side of the endothelial cells, cross the endothelium and infiltrate the subendothelial space where they can get trapped, and this is increased by abnormally elevated concentrations of plasma lipoprotein levels, as well as the integrity of the endothelium. The ApoB-containing lipoproteins that inter the intima can interact with the extracellular matrix, especially with proteoglycans, get retained, and start to accumulate[3,10,11].

Trapped lipoproteins are modified in situ by proteases, lipases, oxidizing agents and enzymes[7,12,13]. Resident macrophages take up modified lipoproteins by phagocytosis or via specific scavenger receptors (such as CD36). Resident macrophages and damaged endothelium release cytokines and chemokines, increasing local inflammation and promoting the recruitment of circulating monocytes at the inflammatory site. This is the starting point of the formation of the atherosclerotic lesion, which is the build-up of a fatty plaque in a highly pro-inflammatory environment, in the artery wall.

Inflammation and lipid accumulation leads to even more modifications, in a vicious circle manner. In addition to that, modified and aggregated lipoproteins display an increased affinity to proteoglycans, and their uptake induces the conversion of macrophages into lipid-filled cells or foam cell[10], the main constituent of early macrophage-rich lesions (i.e. fatty streaks)[14].

Apoptotic macrophages and apoptotic bodies are effectively cleared by efferocytosis, preventing the formation and accumulation of necrotic bodies, and resulting in a non-inflammatory response. However, as the disease progresses, the continuous accumulation of modified lipoproteins increases the apoptosis of macrophages, resulting in defective efferocytosis and autophagy (i.e., regulated recycling process). In advanced lesions, faulty efferocytosis results in necrosis of uncleared apoptotic macrophages and non-resolving inflammation[15–17].

Cell death is at the root of the necrotic core formation. Although apoptosis has been extensively studied in the context of atherosclerosis, recent findings highlight the importance of other types of cell death, including pyroptosis[18] and necroptosis[19,20,21]. Necroptosis is a type of programmed cell necrosis that involves the activation of death receptors by ligands. It has been recently identified as a main contributor of the formation of the necrotic core[19,20,21]. The formation of a necrotic core exacerbates inflammation, sustains lesion progression, and influence the stability of the plaque.

The architecture of the plaque is such that a lipid-rich necrotic core is covered by a fibrous cap. Ultimately, the growth of the core causes the arterial lumen to narrow to the extent where blood flow is impaired. The stability of the plaque depends notably on the thickness of the fibrous cap. A thin fibrous cap is prone to rupture and generates a thrombus that can reach a coronary artery, where it may completely obstruct the blood flow, causing a myocardial infarction. Alternatively, the thrombus can be released in the circulation and reach e.g. the brain where it might cause a stroke[3].

1.3 Lipoproteins

Lipoproteins are spherical vehicles made of proteins and lipids that carry dietary and endogenous lipids in the circulation. Their main purpose is to deliver lipids to peripheral tissues for energy production and to fulfil cellular cholesterol need. After a meal, dietary triglycerides (TGs) are hydrolyzed into glycerol and fatty acids (FAs) in the small intestine, to be reassembled as TGs and packed into chylomicrons in the enterocytes. Chylomicrons are the largest in size (75 to 600 nm) and less dense lipoproteins[22]. They leave the small intestine and enter the bloodstream through lymphatic vessels, to deliver exogenous TGs to peripheral

tissues. In the circulation, chylomicrons are metabolized into chylomicrons remnants, then go to the liver where they contribute to the synthesis of very low-density lipoproteins (VLDLs). VLDLs are generated constantly by the liver to carry endogenous triglycerides in the circulation, and are smaller than chylomicrons, with an average diameter of 30 to 90 nm[23].

Lipoprotein lipase (LPL) is a key enzyme in TG metabolism. LPL sits at the surface of the endothelial cells, on the luminal side of the vessel, and remodels the core of chylomicrons by converting TG to monoacylglycerol and two FAs. It is expressed in the heart, muscle, and adipose tissue, but not in the liver. Released FAs can be transferred to cardiac or squeletic cells that are close by to produce adenosine tri-phosphate (ATP), or stored in adipose tissue[24]. Chylomicrons and VLDLs are metabolized by LPL in the circulation and their gradual conversion produces remnants that are smaller and denser than their parents.

Further conversion of VLDLs leads to the formation of intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDLs), that are very small and dense, containing mostly esterified cholesterol in their core. High-density lipoproteins (HDLs) are secreted by both the intestine and the liver and are taken up by the liver. The transport of cholesterol from peripheral tissue to liver is qualified as reverse cholesterol transport. HDL also undergo transformations in the circulation[25].

ApoB containing lipoproteins are atherogenic and associated with an increased CVD risk. LDL particles are the main cholesterol carrier in humans and their importance in ASCVD is generally accepted, as extensive research showed that LDL-cholesterol is a causal and modifiable risk factor for ASCVD[26,27]. LDL has been indeed considered the most harmful because it is the most represented lipoprotein in the fasting blood, and it has a long half-life in comparison with other ApoB containing lipoproteins.

1.4 TRLs are the major triglycerides carriers in the circulation.

In addition to LDL, there is growing recognition that triglyceride-rich lipoproteins (TRLs) contribute to atherosclerosis[28]. TRLs form an heterogenous class of lipoproteins and are defined according to their size, density, and in an even more specific way, their proteome. TRLs are

chylomicrons, VLDLs, IDLs, and their remnants, or in other words all lipoproteins except LDLs and HDLs.

Triglycerides are the association of three fatty acids with a molecule of acyl glycerol, to be stored mainly in the adipose tissue, or transported in the circulation by TRLs. TRLs are rich in triglycerides in comparison with LDLs and HDLs.

While chylomicrons are produced after a meal, VLDLs are secreted also during fasting, but as we are in a postprandial state most of the time, a relatively high amount of TRLs are constantly circulating in the body. The habit of snacking definitely causes chylomicrons and VLDL levels to go up all the time. TRLs, including chylomicrons remnants, can enter the intima and be taken up by vascular macrophages.[29]

Obesity and insulin resistance are nowadays very common as one Finn out of three is obese, according to the Finnish institute for health and welfare (THL, 2020). In obesity, levels of circulating non-esterified fatty acids (NEFAs) also called free fatty-acids (FFAs) are increased[30,31]. In patients with obesity and particularly insulin resistance, VLDLs are oversecreted and cleared more slowly compared with insulin-sensitive individuals. In addition, inefficient lipolysis of TRLs seen in obesity and metabolic syndrome increases their duration of circulation[32].

High levels of circulating TGs as a cause for ASCVD has been controversial, but epidemiological and genetic studies have shown that TGs and TRLs cause additional ASCVD risk, which has been described as "residual ASCVD"[33,34,35]. High TG serum levels are a risk factor for CVD when other factors like LDL-C are optimally controlled. The combination of raised LDL-C and elevated TG levels constitutes an especially high risk for coronary heart disease[36].

As of today, the mechanisms related to TRLs-induced lipid accumulation and inflammation are not clear. Especially interesting is the potential role of the lipid constituents of the TRLs. Multiple study designs reinforce the hypothesis that TRLs are a causal risk factor for atherosclerosis due to their pro-inflammatory action[37]. As TRLs are big in size, they carry a lot of cholesterol, called remnant-cholesterol, which is a risk factor itself for the initiation and progression of ASCVD. In addition, TRLs carry a lot of TGs, and even though the atheroma is

made of cholesterol, the lipolysis of trapped remnants may contribute strongly to inflammation. Indeed, NEFAs are damaging for the cell and induce the production of inflammatory mediators[38–40]. A key feature of TRLs is that they do not need to be modified to be taken up by macrophages, unlike LDLs[14,37]. Overall, these findings mean that TRLs or remnants may contribute greatly to inflammation.

1.5 Inflammation

Inflammation is a key factor in atherosclerosis and is present at all phases. Inflammatory plaques are especially unstable[3]. Monocytes are recruited at the inflammatory site for clearance of modified lipids and lipoproteins, attracted by cytokines and chemokines released by the damaged endothelium and by intimal cells such as macrophages. At the lesion site, monocytes differentiate into macrophages. Macrophages are innate immune cells that are specialized in the detection, phagocytosis, and neutralization of harmful pathogens. They can secrete cytokines that regulate inflammation. Macrophages can take up lipids and store them in lipid droplets. It is established that LDLs uptake is mediated through a group of structurally unrelated scavenger receptors[41], also referred as molecular pattern recognition receptors, after they have been oxidized[42]. However, it is not clear how TRLs are processed.

Little is known about how macrophages take-up and process TRLs. In contrast with LDLs, native (not modified) TRLs can be taken up by macrophages. It has been recently investigated and demonstrated that their uptake is LPL-dependent[43], and their internalization by macrophages is carried out by caveolae-mediated endocytosis. It has also been shown that TGs are hydrolysed in the lysosome and excreted as FAs. Not much else is known.

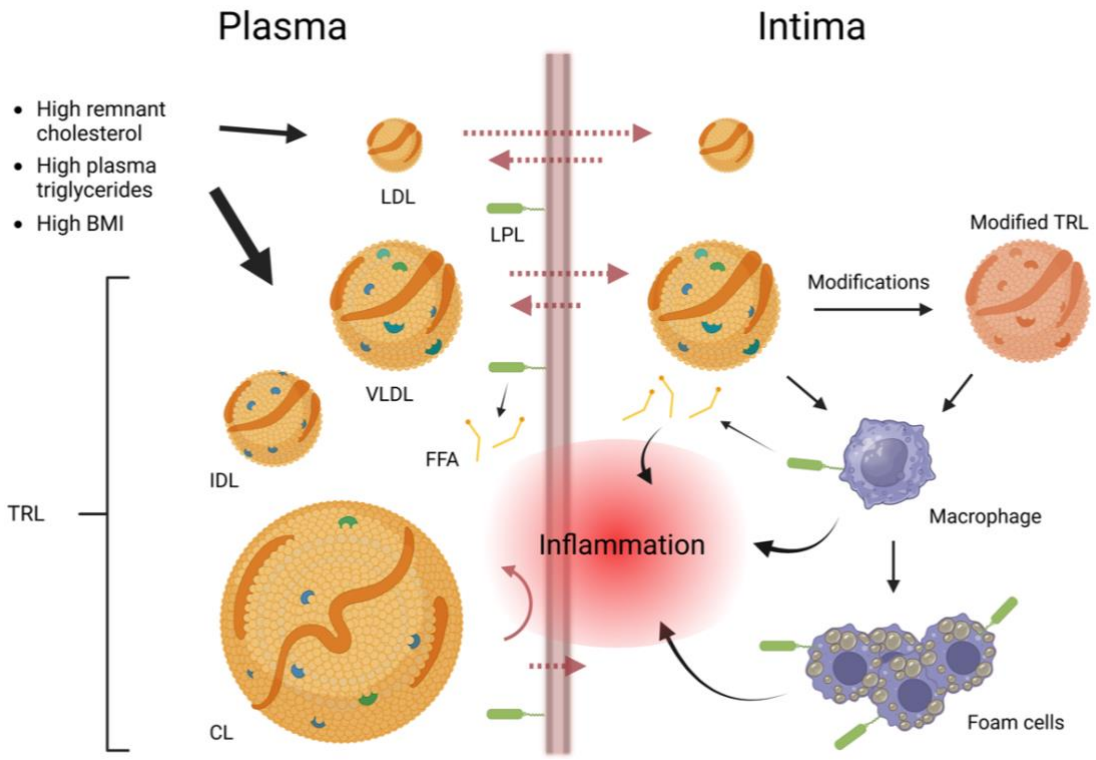
The effect of native TRLs and modified TRLs on inflammation is still unclear. However it is known that LPL is expressed in foam cells and macrophages at the atheroma[37]. Even more important, LPL may promote foam cell formation from TRLs[44].

Activated macrophages produce predominantly proinflammatory cytokines that are involved in the up regulation of inflammatory reactions. VLDL remnants treated with LPL display an increased expression of TNF-α and IL-1β over native VLDLs[45]. TNF-α and IL-1β are pro-inflammatory molecules and cell activation markers that have been linked to ASCVD. Being

able to reduce inflammation at the atherosclerotic lesion may provide opportunities for clinical intervention in the prevention and treatment of ASCVD, as shown by CANTOS trial[46]. Interestingly, the aggregation-susceptibility of LDL, increased by modifications, has been linked with future CV events in patients with atherosclerosis[47,48].

Trapped lipoproteins in the intima undergo modifications by a variety of oxidizing agents and enzymes. Local cells secrete proteases and lipases that modify the structure of the lipoproteins inducing their aggregation, fusion, and retention in the arterial wall[3,10,13,49]. Modified lipoproteins contribute greatly to inflammation in the arterial wall during atherogenesis[14]. Atherosclerotic lesions contain modified LDLs that display extensive signs of oxidation, lipolysis, proteolysis, and are often aggregated[12].

Little is known about the importance of TRL modifications in the development and progression of ASCVD. More specifically, their pro-inflammatory effects may particularly be relevant in the gradual onset and progression of the disease.



Triglyceride-rich lipoproteins accumulates in the arterial wall and induce inflammation, contributing to the onset and progression of atherosclerotic cardiovascular disease. BMI indicates body mass index; CL, chylomicron; FFA, free fatty-acid; IDL, intermediate-density lipoprotein; LPL; lipoprotein lipase; LDL, low-density lipoprotein; TRL, triglycerides-rich lipoprotein; and VLDL, very low-density lipoprotein. Created with BioRender.com, adapted from Nordestgaard (2016)[37].

2 MATERIALS AND METHODS

2.1 Isolation and purification of TRLs

Human chylomicrons, VLDLs (d = ≤ 1.006 g/mL), and IDLs (d = 1.006-1.019 g/mL) were isolated from pooled plasma of healthy volunteers obtained from Finnish Red Cross Blood Service, Helsinki, Finland) by flotation density ultracentrifugation[50] (type 50.2 Ti Fixed-Angle Rotor, 50 000 rpm, 302 000 x g, 12 x 39 mL. Beckman Coulter Optima L-70, USA) for 22h, at a speed of 40 000 rpm at 4 °C in the presence of 3 mmol/L of EDTA[50,51], 100 ug/mL Gentamicin, and KBr. After isolation, TRLs were dialyzed using a semi-permeable membrane (12-14 kDa) and large volumes of cold PBS frequently changed, to be in agreement with the Fick's law of diffusion. TRLs were then concentrated by centrifugation (80 min, 2700 x g, 8 °C) in centrifugal filter units (Amicon® Ultra-15, Sigma-Aldrich, Germany) and purified by sterile filtration (Minisart® syringe filter 0.22 μm, Sartorius Stedim).

2.2 Quantification of lipoproteins

The amounts of TRLs were expressed in terms of their protein concentrations, determined by the method of Lowry et al. using the Pierce™ BCA protein assay kit (Thermo Fisher Scientific, USA).

2.3 Emulsion particles synthesis

In a first time, lipids were extracted from isolated TRLs, dried and resuspended in the appropriate solvent. In a second time, emulsion particles (EPs) were synthesized by sonication.

2.3.1 Lipid extraction

Lipids were extracted in KIMAX tubes (DWK Life Sciences, USA) from isolated TRLs using the Folch's extraction procedure developed in 1957 and still in use[52]. The traditional solvent system is biphasic with a ratio 2:1 Chloroform/Methanol (v/v). We used a slightly different solvent system, consisting of a mixture of Chloroform/Methanol/0,88%KCl in water and in a volumetric ratio of 8:4:3 (v/v/v). Lipids were extracted from 13 mL of TRLs. For 1000 μL of sample, 1250 μL of chloroform and 1250 μL of methanol were added, vortexed, put on ice for

15 minutes, and centrifuged for 5 minutes at a speed of 1700 x g, at room temperature. The supernatant was collected in a clean KIMAX tube. Then, 1250 µL of chloroform and 1250 µL 0,88% KCl in water were added, vortexed, and centrifuged under the same conditions as before. Beneath the aqueous phase lies the lipid-containing organic phase which was of interest. Organic phase is collected with a glass Pasteur pipette without disturbing the biphasic interface. The collected lipid phase was dried using nitrogen gas. The use of inert gases such as nitrogen protects the lipidic compounds from oxidation. Finally, dried pure lipids were reconstituted into 13 mL of PBS (+/+) (1 mL reconstitution for 1 mL sample used). Glassware was washed meticulously with 1:1 ethanol firstly, methanol secondly, and finally sonicated using an ultrasonic bath.

2.3.2 Emulsification by sonication

EPs were created from pure lipids extracts resuspended in PBS (+/+) using an ultrasonic probe (sonicator Branson 250 Sonifier). Lipids (13 mL) were sonicated in a 50 mL Eppendorf tube for 6 rounds of 5 minutes continuous sonication round (output control 3, 40% duty cycle). During the sonication rounds the tube was kept in a 40 °C warmed bath of pure and sterile water. The total sonication time was 30 minutes. Between each round the sample was removed from the water bath and allowed to cool down at room temperature for around 3 minutes, or put on ice if the temperature has risen above approximately 60 °C. After sonication, synthesized EPs were concentrated by centrifugation for 80 min, at 2700 x g and at 8 °C, resulting in the obtention of a total volume of 2 mL concentrated EPs.

2.3.3 Extrusion

The Mini-Extruder (Avanti® Polar lipids, USA) for liposome preparation was used as a first attempt to create EPs. Resuspended lipids were warmed in a water bath for 30 min prior to the use of the extruder. The warm heating block was set at several different temperatures as the optimal temperature for the synthesis of EPs was not known. One of the two syringes was charged with extracted lipids in buffer. The other one was empty and inserted on the other side of the extruder. The sample was passed through the membrane 5 times minimum. As the particles were formed, the sample became clearer.

2.4 Characterization of TRLs/EPs

2.4.1 Size

The size of the nanoparticles (VLDLs, EPs, modified VLDLs, and modified EPs) was measured using the "Zetasizer Nano instrument" (Malvern® Instruments, UK) which can determine the particle size by photon correlation spectrometry, also called dynamic light scattering. Disposable polystyrene cuvettes were filled with 40 µL of sample and discarded after use. The device performed 3 repeated measurements at 20 °C.

2.4.2 Lipids: triglycerides and cholesterol

The quantitative determination of lipids consisted in the measurement of cholesterol and triglycerides content and was performed using commercially available bioassays kits from Thermo Scientific (Vantaa, Finland, reference 981812 and 981301, respectively). The standard used for cholesterol assay was HDL/LDL-cholesterol calibrator 4.835 mmol/L (reference DZ130A-S1V) from Diazyme laboratories. The standard used for TG assay was Precimat Glycerol calibrator 2.29 mmol/L or 21 mg/dL (reference 10166588 Lot 694 996-01) from Roche.

2.5 Cell culture

THP-1 were cultured as monocytes, in which state they were in suspension (non-adherent cells) in T75 cell culture bottles. Cells were grown at a density of 300 000 cells/mL and at a maximum of 800 000 to 1 million cells/mL. Half of the growth medium was changed every 48h, and only once a week cells were centrifuged, old medium being removed and replaced by new growth medium. Cells were maintained in RPMI medium supplemented with FBS (10%), 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM Glutamax (L-Glut), and 25 mM HEPES in a humidified atmosphere of 5% CO_2 at 37 °C.

2.6 TRLs and EPs modifications

2.6.1 Modification 1: Lipolysis with PLA2

935 µL of TRLs (1 mg/mL protein, 2.56 mmol/L TG, 1.17 mmol/L cholesterol) and EPs (2.52 mmol/L TG, 0.59 mmol/L Cholesterol) were incubated at 37 °C for 30 min with 25 µL PLA2-coupled NHS-activated beads stored in PBS +/+, in the presence of 0.04% FA-free bovine serum albumin. The reaction mix was rotating at a very slow rate during the incubation. The samples were then spinned and supernatants were collected and aseptically filtered. The degree of lipolysis induced by PLA2 modification was determined by measuring the release of FAs with the NEFA assay kit (Diagnostic Systems ref 1 5780 99 10 065 kit lot 60140159).

2.6.2 Modification 2: Oxidation

TRLs (1 mg/L protein, 2.56 mmol/L TG, 1.17 mmol/L Cholesterol) and EPs (2.52 mmol/L TG, 0,59 mmol/L Cholesterol) were oxidized by the addition of copper sulfate (5 µM) at 37 °C[53]. Oxidation was terminated by adding EDTA (50 µM). The thiobarbituric acid reactive substance (TBARS) assay was used to measure lipid peroxidation. It detects the level of malondialdehyde (MDA), the major lipid oxidation product, and also some minor related compounds.

2.6.3 Modification 3: Vortexed

TRLs were vortexed at full speed for 30 seconds.

2.7 Cell treatments

Cell experiments were performed on THP-1 differentiated into macrophages. To do so, THP-1 on the monocyte state were seeded at 300 000 cells per mL into duplicate wells of 24-well plate. The differentiation monocyte-to-macrophage was induced by adding 100 nM phorbol 12-myristate-13-acetate (PMA) (Calbiochem cat no. 524400-1MG, Billerica, MA USA) in growth media for 48h, followed by a rest day in fresh growth medium for 24h (72h total differentiation time). Macrophages were incubated at 37 °C with a treatment for 3h or up to 18h. Upon termination of the treatment, cells and supernatants were collected. 50 µL treatments were prepared in PBS (+/+) and added to serum-free growth medium for a total volume per well of 500 µL.

Cells were put in serum-free growth medium (negative control) [1]; FFA-free BSA (0.004%) [2]; EDTA (5 μM) [3]; VLDL (0.26 mmol/L TG) [4]; EP (0.25 mmol/L TG) [5]; OxVLDL (0.26 mmol/L TG) [6]; OxEP (0.25 mmol/L TG) [7]; VxVLDL (0.26 mmol/L TG) [8]; VxEP (0.25 mmol/L TG) [9]; PLA2-VLDL (0.24 mmol/L TG) [10]; and PLA2-EP (0.24 mmol/L TG) [11].

2.8 Cell viability

Cell toxicity was evaluated by measuring lactate dehydrogenase (LDH) activity released from the cytosol of damaged, dead, or lysed cells into the supernatant using a commercially available kit (Roche Diagnostics). For each sample, 50 μL of supernatant was collected at the end of the incubation time and mixed with 50 μL reaction mix (Dye : catalyst = 45 : 1) on a 96-well plate, and run as duplicate. The negative control (lowest cytotoxicity) consisted in fresh experimental medium. The positive control (highest cytotoxicity) was obtained by adding 500 μL triton 1% in experimental medium to a well containing non-treated cells. Absorbance was measured at 490 nm after incubation in the dark, until the high control gets dark red. Cytotoxicity was less than 15% in the experiments.

2.9 Indirect ELISA

Cell-free supernatants were collected to quantify the presence of markers of inflammation (IL-1β and TNF-α) released by THP-1 using mouse/human IL-1β and TNF-α ELISA kits following manufacturer's instructions (DuoSet® ELISA from R&D Systems, Inc). The samples and standards were run in duplicate and actual concentrations were estimated based on a standard curve at known concentration of IL-1β and TNF-α.

2.10 Statistical analysis

Data are presented as mean ± standard deviation. Data was analyzed by two-tailed unpaired Student's t-test for two-group comparisons and one-way ANOVA for comparison of multiple groups with Dunnett's multiple comparisons tests using GraphPad, Prism GraphPad Software, La Jolla, CA).

3 RESULTS AND DISCUSSION

3.1 Synthesis of EPs by sonication generated from human TRLs

The main goal of this project was to study the effect(s) of lipids on inflammation in the context of atherosclerosis. It is not known if the pro-inflammatory action of TRLs is caused by their lipidic components only or mainly. We decided to use EPs generated from human TRLs as a model of lipids-only human TRL-like nanoparticles.

In this study, the terms VLDL and TRL are interchangeable as the isolated TRLs were mainly VLDLs. The synthesis of EPs from LDL lipids extracts has been previously performed[54]. Nevertheless, according to our knowledge, the manufacture of EPs from VLDL lipids extracts has never been performed before.

The first attempt to synthesize EPs used the mini-Extruder Extrusion Technique (Avanti® Polar lipids, USA) for liposome preparation. The main advantage of using this device was the obtention of highly homogenous EPs in size. However, only 1 mL of EPs could be obtained, and the device leaked frequently resulting in the loss of the final product. Therefore, we decided to perform the synthesis by sonication.

Sonication refers to the process of applying sound energy to agitate particles or discontinuous fibers in a liquid (Carbon composites, second edition, 2017). The stability of the prepared nanofluids is greatly dependent on the time of sonication, and the time required to sonicate using a bath is much greater than using a sonicator (Brooker et al., 2008). Thus, we decided to create EPs using a sonication probe. A variety of sonication times were tested in order to obtain the appropriate size, meaning a size as close as possible to the parent TRLs, from which lipids have been extracted. Different conditions were tested to optimize the method. We found that the most effective way was to keep the sample in a warm bath (around 40 °C) during the sonication and let it cool down between each round of 5 min, for around 3 min.

We monitored the size of EPs using dynamic light scattering with the Zetasizer Nano Instrument. Rapidly, it has been determined that the first measurement should be done after 15 min sonication time. Prior to 15 min, the sample did not meet the quality criteria, meaning that it contained highly heterogenous populations of particles. After 15 min, one measurement every

5min sonication round was taken i.e., at 20, 25, and 30 minutes of sonication. As shown in **Fig. 1**, the size of the EPs decreased linearly with the sonication time. However, past 30 min of sonication, sample evaporation became substantial, and the nanoparticles did not get any smaller. At 30 min sonication time, particles were uniform in size, at the end measuring 172 nm.

After 15 min of sonication there were two peaks thought to be EPs (peak width 170.2, intensity 93.8%) and very large particles (peak width 1132, intensity 6.2%). At 30 min sonication, EPs were more homogenous in size (peak width 101.9, intensity 94.5%), whereas very large particles had evolved into two groups of large particles, more uniform in size, represented by two peaks (peak width 654.8, intensity 3.4% and peak width 853.5, intensity 2.1%). Over time, particles became smaller and more homogenous as shown by peaks that display a reduced peak width and a higher intensity.

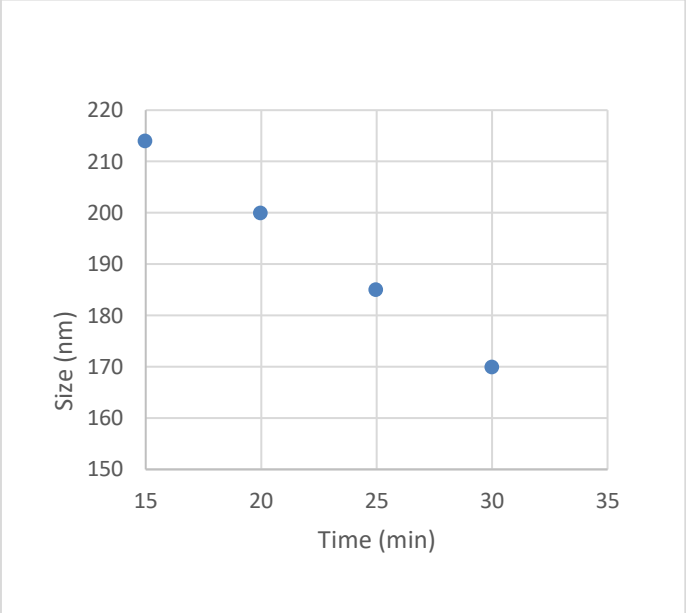


Figure 1 – Emulsion particles synthesis by sonication from TRL lipid extract. Lipids were extracted from TRLs using the Folch's extraction procedure, dried, and reconstituted into PBS (+/+) to be sonicated for 30 min (6 x 5 min). Size was measured at 15 min (215 nm), 20 min (200 nm), 25 min (185 nm), and 30 min (172 nm) of sonication. Representative data from one experiment. Please, note that the axes do not begin at zero.

The size distribution of the TRLs and EPs is shown in Figure 2. The average sizes of them were 91 nm and 170 nm respectively. EP population is more heterogenous than their parents VLDL, as it can be seen with their slightly larger peak width. In addition, we can notice the presence of very large particles in EP mixture represented by very low intensity peaks on the right side of **Figure 2.B**. These very large particles are probably lipidic nanoparticles that did not undergo sonication. However, their proportion is very small.

When the volume to sonicate was less than 4 mL, the sample overheated and evaporated if sonicated any longer. It was also important to centrifuge the samples after synthesis to remove any metallic impurity released from the probe. The probe was cleaned before and after each use to maximize sample quality.

One main limitation of this method was the obtention of a sufficient amount of lipids extract. The lipid extraction from TRLs was tricky, as an important volume of lipids was necessary to synthesize EPs, and only 1 mL of sample could be extracted per KIMAX tube.

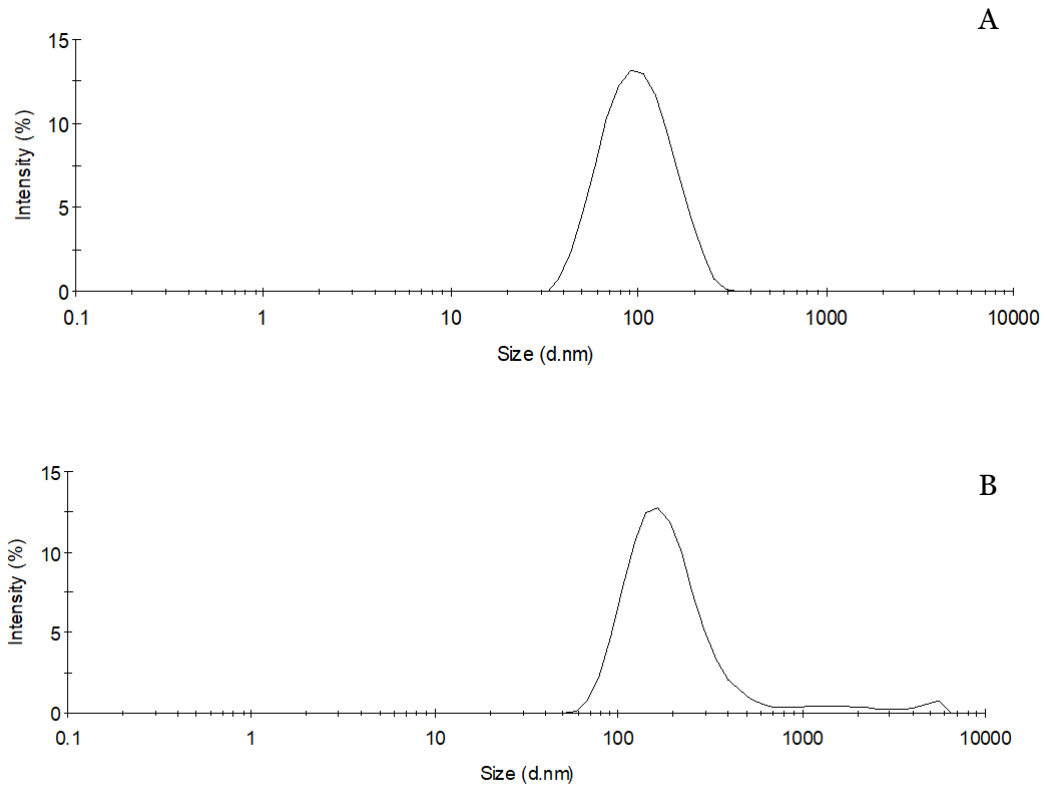


Figure 2 – Size distribution of isolated human TRLs (A) and emulsion particles (EP) (B). Human TRLs (VLDL (d = ≤ 1.006 g/mL) and IDL (d = 1.006-1.019 g/mL)) were isolated from pooled plasma of healthy volunteers obtained from Finnish Red Cross Blood Service, Helsinki, Finland by sequential ultracentrifugation as described in the methods. Emulsion particles were created by sonication using lipids extracts generated from the isolated TRLs. The size of TRLs (A) and EPs (B) was measured using the "Zetasizer Nano instrument", 91 nm and 170 nm diameter respectively. Representative data from one experiment.

3.2 Characterization of TRLs/EPs lipids: cholesterol and triglycerides concentration

The amounts of EPs were expressed as their TGs content. As EPs do not contain proteins, we decided to measure their concentration through lipid quantification, here being TGs. TGs are hydrolyzed by lipase to glycerol and FAs, the amount of which was determined. The method used to quantify cholesterol content measures both esterified and unesterified (i.e., total cholesterol). Manufactured EPs contained twice more TGs than their parent VLDLs (5.04 mmol/L and 2.56 mmol/L respectively). It came in handy as by dilution we could obtain a

comfortable final volume of EPs. Interestingly, EPs and VLDLs contained the same amount of cholesterol (1.17 mmol/L).

We found that sonication led to a different ratio of TG to cholesterol than what we had at first, in the VLDLs. We do not know why the ratio was changed and this needs to be studied. One possible explanation is that we experienced a loss of cholesterol during the extraction, sonication, or concentration processes. Another reason can be that the cholesterol or TGs measurement were not accurate enough.

3.3 Investigation of the release of IL-1β and TNF-α by macrophages treated with TRLs and EPs

THP-1 is a monocyte isolated from the peripheral blood of a patient with acute monocytic leukaemia. It is widely used to study the function and regulation of macrophages in the cardiovascular system[55,56]. Primary monocyte-derived macrophages can display significant inter-individual variation and a relatively short time frame when experiments are doable. As THP-1 is known to behave relatively well similarly to primary monocyte-derived macrophages, it is a good model for this study. Moreover, this project is meant to be a control for other experiments, which are performed with THP-1 cells.

Figure 3. describes the secretion of IL-1β and TNF-α by THP-1 cells when treated with TRLs (mostly VLDLs) versus EPs, for two different incubation timepoints, 3h and 18h.

3.3.1 IL-1β secretion by THP-1 cells when treated with EPs versus treated with VLDLs

As shown on the left panel of **Figure 3.**, IL-1β secretion was clearly increased at 3h for all treatments and control cells, followed by a reduction of concentration in the cell culture medium at 18h incubation time ($p > 0.05$), concentrations that stayed higher than baseline levels. Cells treated with VLDLs did not show an increased release of IL-1β compared with control. We found that cells treated with VLDLs, and control cells released IL-1β similarly at all timepoints ($p > 0.05$). However, levels of IL-1β were increased after 3h incubation with EPs compared with control cells ($p < 0.05$) and cells incubated with VLDLs. At 18h incubation time, levels of IL-1β were similar for EP and VLDL treatments ($p > 0.05$), IL-1β secretion decreased very slightly below control levels ($p > 0.05$).

3.3.2 TNF-α secretion by THP-1 cells when treated with EPs versus treated with VLDLs

As shown on the right panel of **Figure 3.**, levels of TNF-α were slightly increased after 3h incubation with all treatments and control cells. At 3h timepoint, cells treated with VLDLs did not behave differently from the control ($p > 0.05$). However, EPs-treated cells released higher levels of TNF-α than control cells ($p < 0.05$). Secretion of TNF-α is higher at 18h than at 3h incubation time when cells were treated with EPs ($p < 0.05$) but lower than control when treated with VLDLs ($p < 0.05$).

According to our data, EPs induced the secretion of TNF-α compared with control (3h and 18h timepoints $p < 0.05$) when VLDLs did not (3h timepoint $p > 0.05$, 18h timepoint $p < 0.05$). However, control cells also released TNF-α, more than cells treated with VLDLs ($p < 0.05$), but less than cells treated with EPs ($p < 0.05$).

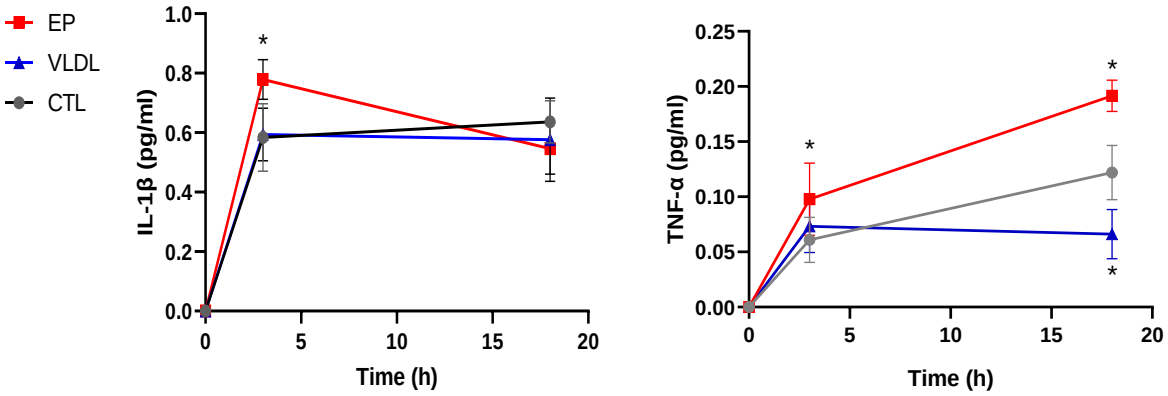


Figure 3 – IIL-1β and TNF-α secretion by THP-1 cells treated with VLDL and EP. THP-1 cells were incubated with VLDL, EP, or PBS+/+ for up to 18 hours. The release of IL-1β and TNF-α was quantified by indirect ELISA at time points 3h and 18h. The data were analyzed using ANOVA with Dunnet's multiple comparison test. * indicates statistically significant differences to control cells at same time point. N=4. EP indicates emulsion particle; VLDL, very low-density lipoprotein; and CTL, control.

Overall, there was no obvious difference in IL-1β secretion between VLDLs-treated cells and control cells, as well as in TNF-α secretion at 3h incubation time. However, at 18h timepoint, cells treated with VLDLs seemed to reduce TNF-α release compared with controls.

According to our results, EPs seemed to trigger inflammation by stimulating the release of TNF-α at 3h and 18h incubation time, and IL-1β at 3h incubation time compared with control.

3.4 Modifications of TRLs and EPs

Figure 4. describes the secretion of IL-1β and TNF-α by THP-1 cells when treated with modified EPs versus unmodified EPs, for two different incubation timepoints, 3h and 18h. The tested modifications are vortexed (VxEP), lipolysis with PLA2 (PLA2-EP), and oxidized (OxEP).

3.4.1 Lipolysis with PLA2

PLA2 hydrolyses phospholipids into lysophosphatidylcholine and NEFAs, promoting the binding of lipoproteins with proteoglycans in vitro[54,57], potentially enhancing their retention in the intima. The action of LDL modified by PLA2 on macrophages promotes foam cell formation[58,59] and PLA2-modified TRLs can induce IL-1β secretion[60].

THP-1 were incubated with FFA-free BSA in PBS as a control for this condition because PLA2-modified VLDLs contained FFA-free BSA. According to NEFA assay, the lipolysis was limited as FFAs levels were similar in PLA2-modified VLDLs (41.4 μM) and PLA2-modified EPs (46.7 μM) compared with native VLDLs (25.4 μM) and unmodified EPs (41.4 μM). We were unsuccessful in modifying TRLs and EPs with PLA2 sufficiently. The amount of NEFAs in the unmodified EPs is surprisingly high, and possibly because of this, the increase in NEFAs is low in the unmodified EPs. Lipolysis did not fail as both PLA2-treated samples have more NEFAs than their native counterparts. Unfortunately, we were unable to identify the cause of limited lipolysis with PLA2. As FFA levels are higher in EPs compared to VLDLs, it might indicate that sonication induces some lipid breakdown.

We can see on the left panel of **Figure 4.** that IL-1β secretion is lower than control cells (unmodified EP-treated cells) at 3h incubation time ($p < 0.05$), to be the same at 18h incubation time. On the right panel, TNF-α release by PLA2-EP-treated cells is identical to control ($p > 0.05$).

3.4.2 Oxidation

The main result of oxidation is the formation of malondialdehyde (MDA) adducts in lysine residues of the proteins and lipid changes. Oxidation decreases interaction of lipoproteins with proteoglycans but increases binding to collagen (decorin-coated) via the action of LPL, responsible for their accumulation at the lesion site. Lipoprotein modification by oxidation has been intensely studied, and it is established now that oxidized phospholipids are pro-inflammatory[61,62.] Oxidized LDL (OxLDL) induce foam cell formation[63,64], the secretion of pro-inflammatory cytokines[65,66], and endothelial cell dysfunction[67]. Notably, extensive oxidation can lead to the fragmentation of apoB[13].

According to TBARS assay, EPs have been oxidized. MDA concentration ([MDA)] in unmodified EPs = 2.78 μM and oxidized EPs (OxEP) = 7.78 μM, giving a ratio [MDA] in oxidized EPs / [MDA] in unmodified EPs of 2.80. However, TBARS assay may not be the best method to measure EPs oxidation. Therefore, other methods should be considered in the future. Because TRLs have not been oxidized, we cannot compare the effect of OxEP and oxidized VLDLs (OxVLDL) on THP-1 cells secretion of IL-1β and TNF-α. However, we focus on the effect of modification by oxidation on inflammation, by comparing the release of IL-1β and TNF-α by THP-1 treated with OxEP with unmodified EPs.

We can see on the left panel of **Figure 4**. that IL-1β secretion is lower than control cells (unmodified EP-treated cells) at 3h incubation time ($p < 0.05$). At 18h incubation time, the secretion is inferior to control ($p > 0.05$). On the right panel of **Figure 4**., TNF-α release by OxEP-treated cells is lower than control at 3h and 18 timepoints ($p > 0.05$). Overall, OxEP do not seem to be more pro-inflammatory than their parents EPs in the context of our experiment. Cells treated with OxEP released less pro-inflammatory cytokines than cells when treated with unmodified EPs. This suggests that uptake of oxidized lipids alone (without proteins) is not sufficient to induce inflammation, and the pro-inflammatory properties of oxidized VLDLs should be studied more in details in the future.

3.4.3 Vortexed

Vortexing lipoproteins and EPs induce their aggregation. VxLDL is a model that has been used to study lipid accumulation in macrophages, leading to foam cell formation[68,69] and have been shown to activate genes involved in inflammation[70].

We can see on the left panel of **Figure 4**. that IL-1β secretion is lower than control cells (unmodified EP-treated cells) at 3h incubation time ($p > 0.05$). At 18h incubation time, the secretion is similar to control ($p > 0.05$). The right panel of **Figure 4**. shows that TNF-α secretion by VxEP-treaded cells is similar to control at 3h timepoint ($p > 0.05$). However, at 18h incubation time, TNF-α secretion is lower than at 3h timepoint, and much lower than in the cells treated with unmodified EPs ($p < 0.05$).

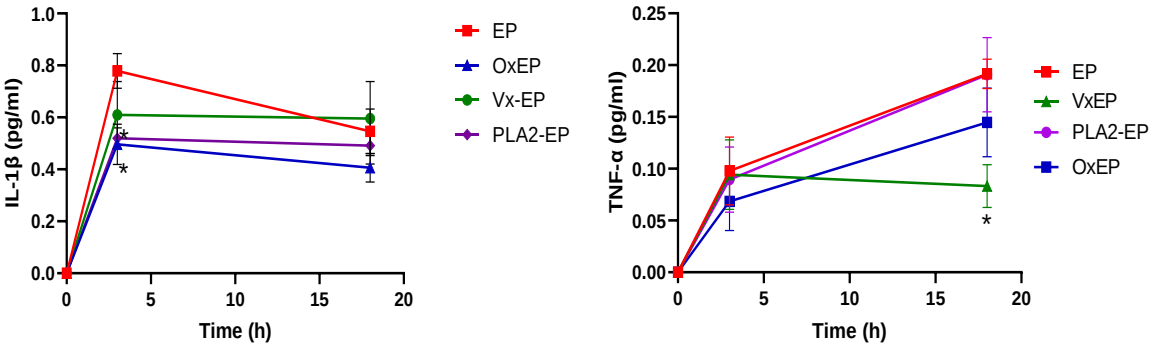


Figure 4 - IL-1β and TNF-α secretion by THP-1 cells treated with modified VLDL and modified EP. THP-1 cells were incubated with VLDL, EP, or PBS+/+ for up to 18 hours. The release of IL-1β and TNF-α was quantified by indirect ELISA at time points 3h and 18h. The data were analyzed using ANOVA with Dunnet's multiple comparison test. * indicates statistically significant differences to cells treated with EPs at same time point. N=4. EP indicates emulsion particle; VxEP, vortexed emulsion particle; PLA2-EP, emulsion particle treated with phospholipase A2; and OxEP, oxidized emulsion particle.

To sum up, levels of IL-1β were increased after 3h incubation with EPs and modified EPs. At 18h incubation time, IL-1β secretion decreased slightly, concentration in the cell culture medium stayed higher than baseline levels. Levels of TNF-α were slightly increased after 3h incubation with EPs and modified EPs. At 18h incubation time, levels were much higher than at 3h incubation timepoint. EPs and PLA2-EP treated cells released TNF-α similarly, OxEP treatment followed the same secretion pattern but with lower concentration in the cell culture medium. Treating with VxEP gave a different effect as TNF-α levels became at 18h incubation time inferior to levels secreted at 3h.

Our results show that EPs promote inflammation by inducing a higher release of IL-1β and TNF-α by THP-1 cells compared with cells treated with TRLs and control cells. High levels of

NEFAs in the unmodified EPs compared to native TRLs could be one reason for the higher activation by EPs than by TRLs, reinforcing the claim that FFAs are pro-inflammatory. Also, it is worth noting that there is a clear difference between IL-1β and TNF-α secretion. This could mean that these two pro-inflammatory cytokines are regulated differently in the context of our experiment. Treating THP-1 cells with unmodified EPs and modified EPs seems to trigger the release of IL-1β which then drops with time, but without returning to baseline levels. In contrast, TNF-α concentration in the cell culture medium increased slightly at 3h treatment but continued to increase at 18h incubation time, except for THP-1 treated with VxEP which levels became smaller than at 3h incubation time. Surprisingly, modification by oxidation did not make EPs more pro-inflammatory compared with unmodified EPs. We hypothesize that oxidized lipids organized as spherical particles do not display pro-inflammatory properties compared with free oxidized lipids.

A serum-free growth medium allowed for the limitation of confounding variables as FBS contains lipoproteins. Normalization was done by subtracting the background absorbance measured from the blank sample which consists of a protein-free buffer.

One limitation of the study was the heterogeneity in size as EPs were usually larger than their parent VLDLs. However, the current approach still allows us to study the effect of the lipids on cells. We think that the quality of lipids is the main factor to take into consideration for the investigation of the role that play the lipids in TRLs-induced inflammation in the context of ASCVD. To our knowledge, this is the first time that EPs were generated from human VLDL lipid extracts. Our work definitely brings something new as the synthesis of EPs preserved the diversity of lipids inherent to physiological human lipoproteins.

Another limitation was the heterogeneity in size as EPs were usually larger than their parent VLDLs. However, we think that the composition of lipids matters more. The quality of lipids is the main factor to take into consideration for the investigation of the role that play the lipids in TRLs-induced inflammation in the context of ASCVD. To our knowledge, this is the first time that EPs were generated from human VLDL lipid extracts. Our work definitely bring something new as the synthesis of EPs preserved the diversity of lipids inherent to physiological human lipoproteins.

4 CONCLUSION

The aim of this project was to investigate the impact of TRLs on inflammation in the context of atherosclerosis. To do so, we addressed three aims. Firstly, we successfully developed a method for the synthesis of EPs generated from human TRLs (Chylomicrons, VLDLs, IDLs). The sizes were not identical but their content in triglycerides and cholesterol used for the experiments were similar. Secondly, we investigated the release of specific markers of inflammation (i.e., IL-1β and TNF-α) by THP-1 cells treated with TRLs EPs, and modified EPs.

This study concluded that EPs seem to promote inflammation, unlike their modified forms. As modified TRLs have been shown to induce cytokine secretion, the result we obtained with EPs is surprisingly different. Whether it is due to differences in the uptake of TRLs and EPs, it needs to be studied. All in all, it is difficult to conclude on the effect of lipids on inflammation, as VLDLs did not exert an inflammatory effect in our experiment compared with control. In the future, we would need to detect inflammation caused by VLDLs compared with control first, in order to compare further with EPs.

Inflammation is an essential component of the advent of CV disease, as it has been shown that reducing inflammation by blocking cytokine pathways diminishes the risk of stroke and myocardial infarction[71]. Inflammation increases clones of leukocytes in peripheral blood, so it is primordial to study macrophages behavior at the atheroma, and in the circulation.

The identification and understanding of the molecular mechanisms underlying macrophage activation by TRLs and modified TRLs, as well as their uptake at the atherosclerotic lesion will bring new insights for the development of therapy aiming at preventing the formation of the atherosclerotic plaques, stabilizing the plaques, and hopefully even reducing already existing plaques. Even more relevant nowadays, inflammation is the link between CV disease and ageing[72].

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