Saturated fatty acids induce NLRP3 activation through a K⁺ efflux
resulting from phospholipid saturation and Na, K-ATPase disruption
in human macrophages.

M.A. Gianfrancesco^{1,2}, J. Dehairs³, L. L'homme⁴, G. Herinckx⁵, N. Esser^{1,2}, O. Jansen⁶, Y. Habraken⁷, C. Lassence⁷, J. V. Swinnen³, M. Rider⁵, J. Piette⁷, N. Paquot^{1,2} and S. Legrand-Poels¹

¹Laboratory of Immunometabolism and Nutrition, GIGA-Inflammation, Infection & Immunity, University of Liège, Liège, Belgium, ²Division of Diabetes, Nutrition and Metabolic Disorders, Department of Medicine, University Hospital of Liège, Liège, Belgium, ³ Laboratory of Lipid Metabolism and Cancer, Department of Oncology, KU Leuven, Leuven, Belgium, ⁴Inserm, Institut Pasteur of Lille, U1011 - EGID, University of Lille, Lille F-59000, France, ⁵De Duve Institute, SSS/DDUV, ICP, Catholic University of Louvain, Louvain, Belgium, ⁶Laboratory of Pharmacognosy, Department of Medicine/ Pharmacy, University of Liège, Liège, Belgium, ⁷Laboratory of Virology and Immunology, GIGA-Molecular Biology of Diseases, University of Liège, Liège, Belgium.

*To whom correspondance should be adressed

Tel : +324 366 24 34, s.legrand@uliege.be

ABSTRACT

NLRP3 inflammasome was recently shown to play a key role in Western diet-induced systemic inflammation and long-lasting trained immunity in myeloid cells. Saturated fatty acids (SFAs) are sterile triggers able to induce the assembly of the NLRP3 inflammasome in macrophages, leading to IL-1 β secretion while unsaturated ones (UFAs) prevent SFAs-mediated NLRP3 activation. Unlike previous studies, we do not see any ROS or ER stress involvement in SFAs-mediated NLRP3 activation. Rather we show that SFAs need to enter the cells and to be activated into acyl-CoA to lead to NLRP3 activation in human macrophages. However, their β -oxidation is dispensable. Instead, they are channeled towards phospholipids but redirected towards lipid droplets containing triacylglycerol in the presence of UFAs. Lipidomic analyses and Laurdan fluorescence experiments demonstrate that SFAs induce a dramatic saturation of phosphatidylcholine correlated with a loss of membrane fluidity, both events inhibited by UFAs. This SFA-induced membrane remodeling promotes a disruption of the plasma membrane Na, K-ATPase, instigating a K⁺ efflux essential and sufficient for NLRP3 activation. This work opens novel therapeutic avenues to interfere with Western diet-associated diseases such as those targeting some enzymes of the glycerolipid pathway.

1. Introduction

Obesity has become a major public health concern as it promotes insulin resistance and is strongly associated with hyperglycemia, dyslipidemia and hypertension, which together are termed “metabolic syndrome” [1]. These metabolic disorders contribute to higher rates of morbidity and mortality due to the increased risk of developing type 2 diabetes (T2D), non-alcoholic fatty liver disease, cardiovascular diseases and cancer [2]. Moreover, a chronic low-grade inflammation has been observed in abdominal obesity that plays a role in the pathogenesis of obesity-related metabolic disorders [3]. This systemic inflammation results mainly from an excessive accumulation of pro-inflammatory macrophages in adipose tissue [3]. The current theory supports that weight gain and adipocytes hypertrophy induce chemokines-promoted recruitment of circulating monocytes [3]. These monocytes differentiate into macrophages secreting pro-inflammatory cytokines such as $\text{TNF}\alpha$, IL-6 and IL-1 β .

IL-1 β has been shown to be a key contributor to the pathogenesis of T2D mainly by interfering with insulin signaling [4] and playing a critical role in the pathological deterioration of pancreatic β -cell function [5]. IL-1 β production is tightly controlled and depends on two signals. First, a pro-inflammatory priming signal stimulates the transcription of *il-1 β* gene through NF- κ B-dependent pathway leading to the synthesis of the Pro-IL-1 β . Secondly, the Pro-IL-1 β cleavage in its biologically active form is mediated by a large cytosolic multiprotein platform termed « inflammasome » [6,7]. Inflammasomes are usually formed through the interaction between a member of the NLR family (nucleotide-binding oligomerization domain (NOD)-, leucine-rich repeat (LRR)-containing proteins), an adaptor protein ASC (Apoptosis-associated Speck-like protein containing a Caspase recruitment domain) and the Pro-caspase-1. Their assembly is triggered in response to pathogen- and danger-associated molecular patterns (PAMPs and DAMPs) [6,7]. Proximity-induced pro-caspase 1 autoprocessing results in the formation of active caspase-1, which, in turn, can cleave the Pro-IL-1 β and lead to the release of IL-1 β .

The NLRP3 inflammasome is characterized by the sensing of a large array of DAMPs such as extracellular ATP, pore-forming toxins and various crystalline and particulate matter (Alum, cholesterol, monosodium urate, silica, asbestos) [8]. More recently, NLRP3 was also

shown to respond to metabolic danger signals that accumulate during obesity like glucose, ceramide and free fatty acids (FFAs) [9,10]. Thanks to the *Nlrp3*^{-/-} high fat diet (HFD) mouse model, NLRP3 inflammasome has been shown to instigate obesity-induced inflammation and insulin resistance (IR) [11,12]. Our group also highlighted the role of NLRP3 inflammasome in obesity-linked metabolic syndrome by demonstrating caspase-1 activation in visceral adipose tissue macrophages (ATMs) in metabolically unhealthy compared to metabolically healthy obese patients [13].

Due to the very large range of divergent NLRP3 activators, it seems impossible that NLRP3 directly recognizes its agonists. It is generally accepted that NLRP3 rather senses disturbances in cellular homeostasis. Several mechanisms have been proposed to account for NLRP3 activation including potassium efflux, lysosomal destabilization induced by various crystals, reactive oxygen species (ROS) production, mitochondria dysfunction and Ca²⁺ homeostasis disruption [8,14]. However, K⁺ efflux seems to be a common denominator to many NLRP3 activators even if its origin can vary according to the NLRP3 trigger [15–17]. The role of K⁺ efflux has been reinforced since NEK7, a Ser/Thr kinase, has been identified as a factor specifically required for NLRP3 activation and interacting with NLRP3 through a K⁺ efflux-dependent way [17,18].

The important contribution of the NLRP3 inflammasome in the pathogenesis of obesity-linked IR and T2D prompted several groups to study the mechanisms underlying FFA-mediated NLRP3 inflammasome activation [11,12]. Previous studies have demonstrated the inhibition of AMP-activated protein kinase (AMPK) by palmitate (C16:0) in LPS-primed bone-marrow-derived macrophages (BMDMs), followed by the defective autophagy and the accumulation of mitochondrial reactive oxygen species (mROS), presumably by a deficiency in the clearance of dysfunctional mitochondria (mitophagy) [12]. More recently, palmitate and stearate (C18:0) have been proposed to mediate NLRP3 inflammasome activation in LPS-primed BMDCs (bone-marrow-derived dendritic cells) or BMDMs through the activation of IRE1 α (inositol-requiring enzyme 1-alpha), one of the three ER stress sensors [19]. According to these authors, saturated fatty acids (SFAs)-induced IRE1 α activation does not depend on the sensing of unfolded proteins but does occur in response to the accumulation of saturated phosphatidylcholine (PC) [19]. Our group demonstrated the activation of NLRP3 inflammasome by SFAs and its inhibition by the co-treatment with unsaturated fatty acids (UFAs) in LPS-primed human monocytes, human monocytes-derived macrophages (MDMs)

and PMA-differentiated THP-1 cells [20]. Since neither ROS, nor IRE1 α seem to be required for SFAs-mediated NLRP3 inflammasome activation in human MDMs and PMA-differentiated THP-1 cells, we further explored underlying mechanisms. In the present study, we demonstrate that SFAs-mediated NLRP3 inflammasome activation involves a K⁺ efflux resulting from Na⁺, K⁺-ATPase disruption due to the accumulation of saturated PC and loss of plasma membrane fluidity. All these events are prevented by co-treatment with UFAs.

2. Material and methods

2.1 Preparation of FFA solutions

The palmitic acid (#P0500), stearic acid (#S4751), oleic acid (#O1008) were purchased from Sigma. A 100 mM stock solution of sodium salt was prepared by dissolving fatty acids in 0.1 M NaOH at 37°C, 65°C, and 75°C for oleic acid, palmitic acid, and stearic acid, respectively. Stock solutions were aliquoted and stored at -20°C for less than one year. A 5% fatty acid free, low endotoxin BSA (Sigma, #A8806) solution was prepared in RPMI 1640. The FFA stock solution and the 5% BSA solution were mixed together to obtain a 2.5 mM working solution with FFA:BSA molar ratio at 3.4:1. After pH adjustment, the working solution was filtered through a 0.2 μ m pore size membrane filter, aliquoted, and stored at 20°C for less than two months.

2.2 Cell culture and treatment

The THP-1 monocytic cell line (ATCC) (authentication in 2011 by DSMZ, Germany) was cultured between 0.5 and 1.0 $\times 10^6$ cells/ml in RPMI 1640 with glutamine supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, 100 U/ml streptomycin and maintained at 37°C under 5% CO₂ atmosphere. Medium and reagents were purchased from Lonza. THP-1 cells were differentiated with 100 ng/ml of PMA (Sigma) for 24 h, washed with PBS, and kept one night at rest in fresh supplemented medium before stimulation. Primary monocytes were isolated from human peripheral blood mononuclear cells (PBMCs) by using CD14 MicroBeads (Miltenyi Biotec) according to the manufacturer's instructions. PBMCs were obtained by single-step density gradient centrifugation with Ficoll-Paque PLUS (GE Healthcare) from buffy coats (Red Cross, Belgium). Buffy coats were obtained from healthy donors after informed consent, and experiments were approved by the ethical committee of the University of Liege. Monocyte-derived macrophages (MDMs) were obtained by culturing freshly isolated monocytes in RPMI

1640 with 20% FBS and 100 ng/ml of human M-CSF premium grade (Miltenyi Biotec) for seven days. After differentiation, cells were treated with FFAs, Triascin C (Santa Cruz, # sc-200574), STF-083010 (Med Chem Express # HY-15845), trans-ISRIB (Cayman chemicals, # 16258), Ouabain octahydrate (Sigma, # O3125), Nigericin (Enzo life science, # BML-CA421), Antimycin A from Streptomyces sp. (Sigma, # A8674), Etomoxir (Sigma, # E1905), Alum (Thermo Fisher Scientific, #77161), H₂O₂ (Sigma, # H1009), 4μ8C (Axon Medchem, # Axon 1902), Mitotempo (Enzo life science, # ALX-430-150) and Mitosox (InvitrogenTM, # M36008). All these compounds were used at the times and concentrations indicated in figure legends.

2.3 *Radiolabeled stearate flux into lipids*

PMA-differentiated THP-1 cells were cultured in 6 well plates and treated with 200 μM of stearic acid and 2μCi/ml of radiolabeled [1-14C] stearic acid in the presence or absence of 200 μM of oleate for 8 h. After washing of the cells with ice-cold PBS, total lipids were extracted with chloroform/methanol (2:1), followed by 10 min vortexing and centrifugation at 20,000 g for 10 min at 4°C. Supernatants containing the total lipids were evaporated overnight. Dried lipids were resuspended in chloroform and analyzed by thin-layer chromatography (TLC) on TLC Silica gel 60 F254 plates (Merck), using the solvent petroleum ether (b.p. 40–60 °C)/diethylether/formic acid (70:30:1,5). Typhoon 9400TM (Amersham Biosciences) was used to scan storage phosphor screens that have been exposed to the radioactive TLC for 4 hours. Upon laser-induced stimulation, light is emitted from the storage phosphor screen in proportion to the amount of radioactivity in the sample. The resulting digital image allows for quantitation using ImageQuantTM TL v7.0 (GE Healthcare). Lipid fractions were also visualized by anisaldehyde staining. Raw Colza extract and cholesterol were used as the lipids reference substance for lipid species identification.

2.4 *Stearate uptake*

PMA-differentiated THP-1 cells were cultured in 6 well plates and treated with 200 μM of stearic acid and 0,1μCi/ml of radiolabeled [1-14C] stearic acid in the presence or absence of 200 μM of oleate. Cells were washed three times with ice-cold PBS and lysed with 50mM NaOH before radioactivity was counted with liquid scintillation analyser (Packard, Tri-carb 1600TR).

2.5 *Lipidomic analysis*

700 µl of homogenized cells are mixed with 800 µl 1 N HCl:CH₃OH 1:8 (v/v), 900 µl CHCl₃ and 200 µg/ml of the antioxidant 2,6-di-tert-butyl-4-methylphenol (BHT; Sigma Aldrich). The organic fraction is evaporated using a Savant Speedvac spd111v (Thermo Fisher Scientific) at room temperature and the remaining lipid pellet was stored at - 20°C under argon. Just before mass spectrometry analysis, lipid pellets are reconstituted in running solution (CH₃OH:CHCl₃:NH₄OH; 90:10:1.25; v/v/v). Phospholipid species are analyzed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) on a hybrid triple quadrupole/linear ion trap mass spectrometer (4000 QTRAP system; AB SCIEX) equipped with a TriVersa NanoMate (Advion Biosciences) robotic nanosource for automated sample injection and spraying. Phospholipid profiling is executed by (positive or negative) precursor ion or neutral loss scanning at a collision energy of 50 eV/45 eV, 35 eV, -35 eV and -60 eV for precursor 184 (sphingomyelin (SM)/phosphatidylcholine (PC)), neutral loss 141 (phosphatidylethanolamine (PE)), neutral loss 87 (phosphatidylserine (PS)) and precursor 241 (phosphatidylinositol (PI)), respectively. Phospholipid quantification is performed by multiple reaction monitoring (MRM), the transitions being based on the neutral losses or the typical product ions as described above. Lipid standards PC25:0, PC43:6, LPC13:0, LPC17:1, SM30:1, PE25:0, PE43:6, LPE13:0, LPE17:1, PI25:0, PI31:1, PI43:6, LPI13:0, LPI17:1, PS25:0, CER d18:1/17:0, PS31:1, PS37:4, LPS13:0, LPS17:1 (Avanti Polar Lipids) are added based on the amount of DNA of the original sample. Typically, a 3 min period of signal averaging is used for each spectrum. The data are corrected for carbon isotope effects and chain length and analyzed using in house-developed software (RALP 4.0). Only the phospholipid species displaying an intensity of at least 5 times the blank value are taken into account. Each lipid specie is represented as a % of the total lipid content of a specific class.

2.6 *Subcellular fractionation*

5×10⁶ cells were transferred into 250µl of fractionation buffer (20mM HEPES pH 7.4, 10mM KCl, 2mM MgCl₂, 1mM EDTA, 1mM EGTA, 1mM DTT and complete protease inhibitor cocktail (Roche Applied Science)) by scraping and left on ice 15min. Cell suspension was passed through a 25 gauge needle until cells were lysed and left on ice 20min. Samples were centrifuged at 720xg for 5min. Nuclear pellet was discarded and the cytoplasm, membrane and mitochondria containing supernatant was centrifuged at 8.000xg for 5min. Mitochondria

containing pellet was discarded and the cytoplasm and membrane containing supernatant was ultracentrifuged at 100.000xg for 1h. Cytoplasm containing supernatant was stored at -20°C before protein dosage and immunoblotting. Membrane containing pellet was washed by adding 200µl of fractionation buffer and passed through a 25 gauge needle several times before being ultracentrifuged at 100.000xg for 45min. The membrane pellet was resuspended in 50µl of TBS with 0,1% SDS and sonicated for 5min before protein dosage and immunoblotting. All centrifugations were done at 4°C and samples were kept on ice throughout the procedure.

2.7 *Immunoblot analysis*

Total extracts were prepared by scraping the cells in total lysis buffer (160 µl/ 2×10⁶ cells) [87.5 mM Tris-HCl pH 7.5, 75 mM NaCl, 0.5 mM EDTA, 0.5% Igepal, 0.125% sodium deoxycholate, 10% glycerol, 2% SDS, 3% β-mercaptoethanol, 0.002% bromophenol blue, 0.5 mM PMSF, and complete protease inhibitor cocktail (Roche Applied Science)]. After shearing 20 times through a 21 gauge needle, samples were collected at -20°C after a centrifugation to remove Alum in the corresponding treatments. Culture supernatants were also collected at -80°C after a centrifugation to remove cells. Total lysates (30 µl) or supernatants (18 µl) were subjected to SDS-PAGE. After electrophoresis, proteins were transferred from gel to a PVDF membrane (GE Healthcare). The primary antibodies used were as follows: anti-IL-1β propeptide (R&D Systems, #MAB6964), anti-IL-1 β (R&D Systems, #AF-201-NA), anti-HSP90 (Santa Cruz, #sc-7947), anti-αtubulin (Sigma, #T6199), anti-Na,K-ATPase α1 subunit (Abcam, #ab76020), anti-ATF4 (Cell signaling, #11815). The secondary antibodies conjugated to HRP were anti-rabbit (Dako, #P0217), anti-goat (Dako, #P0160), and anti-mouse (Dako, #P0447). The detection was performed with ECL or ECL plus Western blotting substrate (Pierce) by using the digital imaging system ImageQuant LAS 4000 (GE Healthcare).

2.8 *ELISA*

Mature IL-1β was quantified in supernatants by ELISA with IL-1 beta Human Uncoated ELISA Kit (Thermo Scientific) according to the manufacturer's recommendations.

2.9 *Laurdan Two-photon microscopy*

After the treatments, cells were washed three times with PBS and incubated with 2,5 μ M Laurdan in a 5% CO₂ incubator for 1 hour in the dark. Afterwards, two-photon excitation microscopy (Zeiss LSM 510 microscope) was performed at room temperature and Laurdan intensity images were obtained using a Plan-Neofluar 40x/1.3 Oil DIC objective under excitation at 780 nm with a Mai Tai Ti:Sapphire laser (Spectra-Physics). Internal photon multiplier tubes collected 8 bit, unsigned images at a scan speed of 1.6 μ s/pixel. Two Laurdan intensity images were recorded simultaneously with emission in the range of 390–465 nm and 500–550 nm. The measure of membrane fluidity was obtained by using Generalized Polarization (GP) value, defined as: $GP = (I_{440} - I_{510}) / (I_{440} + I_{510})$ where I_{440} and I_{510} are the emission intensities captured with the 390–465 nm and 500–550 nm filters, respectively. The GP formula was applied to each pixel using the two Laurdan intensity images and GP images were pseudo-colored in ImageJ. The colors indicate different GP values following the reported scale.

2.10 Ouabain-sensitive $^{86}\text{Rb}^+$ uptake

After treatment, the cells were incubated in $^{86}\text{Rb}^+$ (PerkinElmer, #NEZ072001MC, 5 μ Ci/ml) containing medium in presence or absence of ouabain (100 μ M) during 30min, washed with PBS and lysed in 0,5%Triton X-100 containing medium before radioactivity was counted with liquid scintillation analyser (Packard, Tri-carb 2100TR). The ouabain-sensitive $^{86}\text{Rb}^+$ uptake is defined as difference between the total $^{86}\text{Rb}^+$ uptake and the $^{86}\text{Rb}^+$ uptake in presence of Ouabain. Counted radioactivity (cpm) of different amounts of $^{86}\text{Rb}^+$ (pmol) was used to make a calibration curve allowing us to determine the quantities of $^{86}\text{Rb}^+$ (pmol) in lysed cells.

2.11 qRT-PCR

Total RNAs were extracted with TriPure Isolation Reagent (Roche) according to the manufacturer's protocol. After quantification by spectrophotometer Nanodrop 1000 (Thermo scientific), 500 ng of RNA were reverse-transcribed by using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific). Obtained cDNA was submitted to qRT-PCR on a LightCycler 480 (Roche Applied Science). Relative gene expression was calculated using the 2-delta delta C method with HPRT1 as housekeeping gene [21]. Results are representative from three independent experiments quantified in triplicate. The following primers were used:

HPRT1-Fw: 5'-TGACACTGGCAAAACAATGCA-3'; HPRT1-Rv: 5'-GCTTGCGACCTTGACCATCT-3',
sXBP1-Fw: 5'- CTGAGTCCGCAGCAGGTG-3' ; sXBP1-Rv: 5'- ACTGGGTCCAAGTTGTCCAG-3'.

2.12 Lipid droplet staining

After treatment, cells were fixed (4% paraformaldehyde, 20 min, 37°C) and permeabilized (0,1% saponin) before staining nucleic acid with Hoechst 33258 (Thermo Fisher Scientific, #AC229891000) and lipid droplet with LipidTOX (Invitrogen™, #H34477) according to the manufacturer's recommendations. Images were acquired using a Leica SP5 confocal microscope (Leica Microsystems) using LAS-AF software. The microscope is equipped with 405nm, Multi-Argon laser, 561nm and 633nm laser lines, three Photomultiplier tubes and one HyD detector. Images were scanned using 63x/1.3 Glycerol objective lense (Magnification/numerical aperture).

2.13 Statistical analyses

All statistical analyses were carried out using GraphPad Prism 6 for Mac OS X (GraphPad Software, Inc.) and presented as means ± SEM. The Student t-test was performed for simple comparison (two groups). Significance is indicated by a symbol.

2.14 Heat Map

Heat map was performed with a web-based tool called Morpheus, a versatile matrix visualization and analysis software available on: <https://software.broadinstitute.org/morpheus/>.

3. Results

3.1 *Neither ROS nor IRE-1 α are involved in SFAs-mediated NLRP3 inflammasome activation in human macrophages.*

We previously demonstrated the NLRP3 inflammasome activation in response to SFAs in human MDMs and PMA-differentiated THP-1 cells [20]. This well-known human macrophages model expressing high levels of Pro-IL-1 β and NLRP3 is very suited to study the second signal leading to NLRP3 inflammasome assembly and allows the secretion of high IL-1 β levels measurable by immunoblot on cell supernatants [20]. In this model, the production of IL-1 β can already be detected after 4h treatment with SFAs concentrations as low as 100

μM and is optimal after 8h treatment [20]. MDMs express low levels of the Pro-IL-1 β and NLRP3 but SFA treatment induces a slight NF- κB -dependent *il-1 β* gene expression in addition to trigger NLRP3 inflammasome assembly leading to the secretion of low levels of IL-1 β that can be measured by ELISA [20].

Since ROS were initially involved in SFAs-mediated NLRP3 inflammasome assembly in LPS-primed BMDMs [12], we proceeded to ROS measurements in FFA-treated human macrophages. However, we did not detect any ROS neither in the cytosol nor in the mitochondria of PMA-differentiated THP-1 cells after treatment with SFAs (200 μM) or UFAs (200 μM) for different times when compared with vehicle alone (BSA), while cytosolic and mitochondrial ROS could be measured after treatment with H₂O₂ and antimycin A, respectively (Fig.1A). As in PMA-differentiated THP-1 cells, mitochondrial ROS were observed in antimycin A-stimulated MDMs but not in SFAs-treated MDMs (Fig.1B).

More recently, Robblee et al. proposed a role of the UPR/IRE-1 α branch in SFAs-mediated NLRP3 activation [19]. As expected, stearate induced the activation of the two UPR branches involving either IRE1 α or PERK, as demonstrated by the accumulation of spliced *Xbp1* mRNA (Fig. 2A, 2B) and ATF4 protein (Fig. 2G), respectively, in both PMA-differentiated THP-1 cells and MDMs. The inhibition of the IRE1 α endoribonuclease activity prevented the *Xbp1* mRNA splicing as shown by the complete or partial loss of *sXbp1* mRNA with 4 μM 4C or STF in both macrophages models (Fig. 2A, 2B). However, none of these inhibitors significantly affected the stearate-mediated IL-1 β secretion, which rules out the contribution of the IRE1 α pathway (Fig. 2C, 2D). ISRIB, a drug known to reverse the effects of eIF2 α phosphorylation, efficiently prevented the accumulation of the ATF4 protein (Fig. 2G) but only slightly down-regulated the stearate-mediated NLRP3 inflammasome activation in PMA-differentiated THP-1 cells (Fig.2E) while only a downwards trend was observed in MDMs (Fig.2F). Altogether, these results demonstrate that neither ROS nor ER stress play a key role in the triggering of NLRP3 inflammasome assembly in response to SFA in human macrophages.

3.2 While SFAs activation into fatty acyl-CoA is required for NLRP3 inflammasome activation, the β oxidation is dispensable for both the SFA- and UFA-mediated effects.

To elucidate the mechanism by which FFAs modulate NLRP3 inflammasome assembly, we further investigated the fate of SFAs when added alone or in combination with UFAs in the

extracellular medium of human macrophages. Some FFAs can bind to membrane G-protein coupled receptors such as FFAR1-4 [22]. The TLR4 was also proposed to mediate some SFAs pro-inflammatory effects [23]. On the other hand, FFA are internalized by flip flop mechanism or through receptors-transporters [10]. Inside cells, FFA can be activated into acyl-CoA by acylCoA synthetase (ACS) to be used in metabolic pathways like β -oxidation in mitochondria or lipid synthesis in ER [24]. Another pool of FFA can be transported to the nucleus and modulate gene transcription through nuclear receptors [25].

In order to test whether SFAs need to be internalized into the cells and activated into acyl-CoA to induce the NLRP3 inflammasome assembly, we used Triacsin C (TC), a competitive inhibitor of ACSs (long-chain fatty acyl CoA synthetases). While TC had no effect on IL-1 β secretion in response to Alum, TC inhibited the release of IL-1 β in response to palmitate and stearate without affecting the Pro-IL-1 β levels in cellular extracts (Fig.3A). To shuttle into the mitochondria and enter the β -oxidation cycle, the acyl-CoA has to be converted into acylcarnitine via carnitine palmitoyltransferase (CPT) 1 [10]. The inhibition of this enzyme with etomoxir did not prevent SFAs-mediated IL-1 β secretion (Fig.3B, 3C). Quite the opposite, preventing the palmitate mitochondrial flux by increasing etomoxir concentrations further stimulated PA-mediated NLRP3 inflammasome activation in a dose-dependent way (Fig 3C).

Since oleate (C18:1) has been proposed to protect skeletal muscle cells from PA-induced apoptosis by promoting palmitate β -oxidation [26], we wondered whether oleate could counteract SFA-mediated NLRP3 inflammasome activation by the same way. As we can observe in Fig.3B, oleate co-treatment prevented stearate-induced IL-1 β secretion but etomoxir had no effect on oleate-mediated inhibition. Altogether, these results demonstrate that β -oxidation does not play any role neither in the SFA-dependent NLRP3 inflammasome activation nor in the oleate-induced inhibition. Accordingly, we turned to the lipid synthesis, the second metabolic pathway involving fatty acyl-CoA.

3.3 Stearate is mainly incorporated in polar lipids but is redirected towards triacylglycerol in the presence of oleate.

We decided to follow the stearate flux by using radiolabeled stearate. The uptake of ^{14}C -labeled stearate by PMA-differentiated THP-1 cells linearly increased with time at least until 12h and was only slightly affected by the presence of oleate, suggesting that the UFAs

inhibiting effect on SFA-mediated NLRP3 inflammasome activation cannot be explained by a competition between SFAs and UFAs for cell internalization (Fig. 4A).

To assess the stearate flux into lipids, we treated PMA-differentiated THP-1 cells with stearate (200 μ M) plus trace amounts of 14 C-labeled stearate for 8h. Total lipids were extracted and labeled diacylglycerol (DAG), triacylglycerol (TAG), cholesterol esters and polar lipids were analysed by TLC. When we focused on the 14 C-labeled stearate distribution among the lipid classes, we could see a major stearate incorporation in polar lipids (62% of the total lipid radioactivity) and to a lesser extent in TAG (27%) and DAG (11%) (Fig. 4B). Interestingly, when 14 C-labeled stearate was combined with oleate, 14 C-labeled polar lipids dramatically decreased (down to 25%), 14 C-labeled DAG disappeared whereas 14 C-labeled TAG significantly increased (up to 72%) (Fig. 4B).

The synthesis of TAG was also assessed by staining the lipid droplets (LD). A few small LD were observed after stearate treatment while oleate alone or combined with stearate triggered the formation of many big LDs confirming its efficiency to induce the synthesis of neutral lipids (Fig. 4C).

Altogether, these results suggest that stearate flux in PMA-differentiated THP-1 cells is mainly channelled towards polar lipids, while it is redirected towards TAG synthesis in the presence of oleate.

3.4 Stearate induces a strong phospholipid saturation correlated to a loss of membrane fluidity, both events prevented by oleate co-treatment.

The different classes of phospholipids such as the phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI) were analyzed by mass spectrometry-based lipidomics. PC and PE are the most abundant phospholipids in all mammalian cell membranes. When we classified the individual species of each class into saturated (no double bond), mono-unsaturated (double bond=1) and poly-unsaturated (double bonds >1) species, we observed a dramatic saturation of PC after stearate treatment (Fig. 5A). In control cells (BSA), most PC species were either mono-unsaturated (49,45%) or poly-unsaturated (40,01%) and only 10% carried no double bonds. The stearate treatment reversed the situation, making almost the half of the PC species saturated (46,35%), leaving only 33,41% and 20,24% of mono- and poly-unsaturated PC species, respectively (Fig. 5A). However, the oleate treatment only induced a slight decrease of saturated PC species

(4,08%) compared to BSA control with a significant increase of poly-unsaturated ones (55,56%) (Fig.5A). Interestingly, the combination of oleate with stearate prevented the strong stearate-mediated PC saturation that even tended towards a decrease (3,72%). The same trend was observed on the saturation profile of the three other classes, PE, PI and PS, whatever much less marked (Fig. 5A).

The heatmap allows to highlight the ratios of the abundance of each phospholipid species in FFA-treated cells over BSA control cells (Fig. 5B). As expected, the majority of the saturated species increased after stearate treatment, specially those with a total number of fatty acid carbons of 34 and 36 and this effect was reversed by oleate co-treatment. Surprisingly, some mono-unsaturated species such as those with 36, 38 and 40 carbons were also up-regulated by stearate and the combination stearate plus oleate but not with oleate alone. Overall, the poly-unsaturated species were down-regulated in response to stearate, mainly the first ones of each saturation class (32 :2, 34 :2, 32 :3, 34 :3, 34 :4, 36 :4, 36 :5, 38 :6,...). This effect was also present but less marked with oleate alone and the combination stearate plus oleate.

Given the large increase in PC saturation after stearate treatment, we investigated its effects on the physical state of membranes by using the Laurdan fluorescence [27,28]. This probe tends to distribute evenly in lipid bilayers but its emission spectrum is influenced by the polarity of its environment ; in apolar environments such as phospholipid bilayers in liquid ordered (Lo) or solid (So) phase enriched in saturated lipid species and cholesterol, its emission maximum is at 440 nm, whereas in relatively polar environments such as membranes in liquid disordered state (Ld) comprising mainly unsaturated lipids, its emission maximum is at 490 nm. Generalised polarisation (GP) images can be constructed from the fluorescence intensity images obtained with blue and green band-pass filters on the microscope, allowing assessment of the phase state of the lipid domains. The GP images show that the PMA-differentiated THP-1 cells are relatively round with quite fluid membranes and some yellow intracytoplasmic vesicles (Fig. 6, BSA). The treatment with stearate (8h) strongly affects the cell morphology, making their plasma membranes much less fluid. The number of vesicles is increased but they appear smaller (Fig.6). However, oleate alone or in combination with stearate does not seem to disturb neither the cell morphology nor the lipid bilayers phase but induces an increase of both the number and the size of the yellow vesicles (Fig.6). Such Laurdan-stained cytosolic vesicles were previously observed in other kinds of cells and were recently shown to be LD that are very apolar organelles [28]. Since we previously observed

the accumulation of LD in PMA-differentiated THP-1 cells after treatment with oleate and the combination stearate plus oleate (Fig. 5C), we can consider these Laurdan-stained cytosolic vesicles like LD.

Altogether, the combined lipidomic and Laurdan fluorescence analyses demonstrated a chemical and physical membrane remodeling in stearate-treated macrophages-like cells, which can be prevented by the co-treatment with the corresponding mono-unsaturated fatty acid oleate.

3.5 A K⁺ efflux is essential and correlated to SFAs-mediated IL-1 β release but prevented by UFA co-treatment.

A K⁺ efflux seems to be a common step to all NLRP3 inflammasome activation settings [15–17]. As expected, increasing extracellular K⁺ concentrations from 5 to 55mM had a dose-dependent inhibitory effect on NLRP3 activation by Alum in PMA-differentiated THP-1 cells (Fig. 7A). The same kind of effect, even more pronounced, was observed in the case of SFAs, demonstrating for the first time that a drop of cytosolic K⁺ concentrations is also a requirement for SFAs-mediated NLRP3 activation both in PMA-differentiated THP-1 cells and MDMs (Fig. 7B). To establish a reliable correlation between intracellular K⁺ concentrations and NLRP3 activation, we measured IL-1 β release and K⁺ efflux in parallel, in time-course experiments. As expected, both the extent and the kinetics of IL-1 β release perfectly matched with the K⁺ efflux in response to Alum, nigericin and ouabain (Na, K-ATPase inhibitor) (Fig. 7C). In comparison to these NLRP3 triggers, stearate induced a lower and slower drop of intracellular K⁺ concentrations, reaching 85% of control cells concentrations after 8h but again the K⁺ efflux fitted with IL-1 β production (Fig. 7D). Interestingly, oleate alone did not lead to a loss of intracellular K⁺ and moreover, it prevented the stearate-mediated K⁺ efflux, which correlated with its lack of effect or its inhibiting effect on IL-1 β production, respectively. Unexpectedly, oleate alone or in combination with stearate even induced a slight increase in intracellular K⁺ concentrations (Fig. 7D). Altogether, these results demonstrate that SFAs induce a K⁺ efflux that is required and correlated with IL-1 β secretion, both events inhibited by UFAs.

3.6 A disruption of the plasma membrane Na, K-ATPase is induced by SFAs and prevented by UFAs co-treatment.

The Na, K-ATPase maintains the Na, K gradient across the plasma membrane by shuttling 2 K⁺ in the cell and releasing 3 Na⁺ outside. Its activity is known to be modulated both by biophysical properties of the plasma membrane and specific interactions with phospholipids [29]. Ouabain, a specific inhibitor of the Na, K-ATPase, leads to a strong K⁺ efflux associated to a significant NLRP3 inflammasome activation (Fig. 7C). For all these reasons, we tested the effect of the different SFA/UFA treatments on Na, K-ATPase activity that we estimated by the ouabain sensitive-⁸⁶Rb⁺ uptake. The activity of the Na, K-ATPase was down-regulated to 92% and 88% with palmitate (200μM, 8h) and stearate (200μM, 8h), respectively, while the Na, K-ATPase α1 catalytic subunit levels in the membrane remained unchanged after the various treatments (Fig. 8A). Furthermore, the ouabain sensitive-⁸⁶Rb⁺ uptake was not affected by oleate alone and even slightly up-regulated with the combination stearate plus oleate (103%) (Fig. 8A). Interestingly, the activity of the Na, K-ATPase seems to be correlated to intracellular K⁺ concentrations shown in Fig. 7D. Both SFAs also disrupted the Na, K-ATPase pump in MDMs ; palmitate (400μM, 8h) and stearate (400μM, 8h) reduced the activity of the Na, K-ATPase to 77% and 76.5%, respectively, without modifying the protein levels in the plasma membrane (Fig. 8B).

4. Discussion

Today, in Western societies, over 80% of deaths are due to non-communicable diseases including those influenced by the consumption of Western-type calorically rich diets (WD) such as type 2 diabetes, obesity and cardiovascular diseases. NLRP3 plays a key role in the development of these WD-associated diseases by sensing sterile danger signals and triggering systemic inflammation. Very recently, a notable study demonstrated that WD, in addition to induce systemic inflammation, instigates a long-lasting trained immunity through NLRP3-dependent myeloid cells reprogramming [30]. In light of these recent data, there is a great interest in understanding how WD activates the NLRP3 inflammasome. SFAs have been proposed as NLRP3 metabolic triggers [10–12]. We also previously demonstrated both the activating effect of SFAs and the protective effect of UFAs on NLRP3 inflammasome activation in human macrophages [20]. Here, we show the important role of the SFAs flux towards phospholipids leading to a loss of membrane fluidity and a K⁺ efflux correlated to the Na, K-ATPase disruption. Interestingly, all these events are prevented by UFAs that redirect the SFA flux towards TAG.

Prior studies have involved mROS accumulation due to impairment of AMPK-regulated autophagy in the palmitate-mediated NLRP3 activation in LPS-primed BMDMs [12]. Others showed mROS production after palmitate treatment of LPS-primed murin peritoneal macrophages but the mitochondrial antioxidant MitoTEMPO failed to inhibit the palmitate-mediated IL-1 β release [31]. We did not observe ROS production neither in PMA-differentiated THP-1 cells nor in MDMs after both palmitate or stearate treatments.

More recently, the IRE1 α -dependent UPR branch was proposed to mediate the SFA-induced NLRP3 inflammasome activation in LPS-primed BMDCs [19]. Indeed, Robblee et al. demonstrated that both inhibitors of IRE1 α endoribonuclease activity (4 μ 8C and STF) decreased the extent to which SFA treatment induced both *Xbp1* splicing and IL-1 β secretion. IRE1 is known to be activated by the recognition of unfolded proteins in ER lumen but compelling evidences suggest that IRE1 is also able to sense ER lipid bilayer stress [10,32]. Authors demonstrated through TLC performed on lipid extracts of radiolabeled palmitate-treated BMDCs that palmitate is mainly incorporated into PC. Limiting PC synthesis by chemical inhibitors reduced the ability of both palmitate and stearate to induce *Xbp1* splicing and IL-1 β secretion, suggesting that incorporation of SFAs into ER membrane PC contributes to the activation of both IRE1 α and NLRP3 pathways. However, the mechanism by which the SFA-induced IRE1 α -XBP1 pathway could lead to NLRP3 inflammasome activation still remains unknown. We also observed a strong and rapid activation of the PERK-EIF2 α -ATF4 and IRE1 α -XBP1 pathways in our both stearate-treated human macrophage models. However, both STF and 4 μ 8C had no effect on stearate-mediated NLRP3 activation neither in PMA-differentiated THP-1 cells nor in MDMs while they induced a significant decrease of *Xbp-1* mRNA splicing. These opposing data concerning involvement of both ROS and IRE1 α could be explained by the use of various macrophage models, either resting or LPS-primed and by the selection of different treatment conditions such as the kind of SFA (palmitate or stearate), the SFA concentration and treatment time. We definitively think that LPS pre-treatment, by rapidly inducing the expression of many genes such as those involved in some metabolic pathways [33], could really influence the FFA signalling inside macrophages.

By using inhibitors of ACSLs (triascin c) and CPT1 (etomoxir), we demonstrated that both palmitate and stearate have to be taken by the macrophages and activated into acyl-CoA but do not need to enter the mitochondria to induce NLRP3 inflammasome activation.

Moreover, preventing palmitate to enter the mitochondria had even an upregulating effect on NLRP3-mediated IL-1 β release. Etomoxir probably induced a redirection of the palmitate flux towards another metabolic pathway like lipid synthesis favoring NLRP3 inflammasome activation. This synergistic effect of etomoxir was only obtained with palmitate and not with stearate. This observation allows us to suggest that, in human macrophages, palmitate is a less efficient NLRP3 trigger than stearate because its flux is more important towards β -oxidation at the expense of the lipid synthesis. Interestingly, this exacerbating effect of etomoxir has been already observed on palmitate-induced inflammatory and ER stress responses in human macrophages [34].

When we followed the incorporation of radiolabeled stearate in lipids, we also observed a major incorporation into polar lipids. But we decided to go further. We undertook a lipidomic analysis to accurately determine the membrane phospholipids composition after the different treatments with SFA and/or UFA. We highlighted a rapid and dramatic increase of saturated phospholipids especially in the case of the PC whose almost 50% became saturated after 8h treatment with stearate compared to 10% in BSA condition. The heatmap revealed an increase of saturated species but also an up-regulation of some mono-unsaturated ones with an overall reduction of the poly-unsaturated species, which looks like *de novo* lipogenesis profile [35]. Indeed, an ongoing analysis of the whole transcriptome of stearate-treated MDMs allowed us to identify an upregulation of some genes involved in *de novo* lipogenesis and glycerolipid synthesis already after 8h treatment. The dramatic increase observed in the saturation of phospholipids could be explained not only by an exogenous stearate uptake but also by increased *de novo* lipogenesis and glycerolipid synthesis. Such hypothesis needs further investigation.

It is well-known that saturated fatty acyl chains, straight and closely packed, create a liquid-ordered phase while unsaturated lipids with a kinked shape lead to a liquid-disordered phase [36]. As expected, we observed, through the Laurdan fluorescence, a radical and rapid decrease of the membrane fluidity of human macrophages. In general, more the acyl chains are saturated, more the phase is liquid-ordered and more the membrane is thick [37]. These biophysical properties can influence the activity of integral proteins [36]. The transmembrane (TM) part of the protein, often composed of one or several α -helices, is poor in polar residues. The length of the hydrophobic core of its TM domain has to match the hydrophobic thickness of the membrane in order not to expose non-polar residues to water. Accordingly, membrane

thickening can induce a conformational modification of the TM domain and thereby can inactivate the protein [38]. On the other hand, the lack of freedom mediated by a loss of membrane fluidity can prevent the TM domain conformational transitions required for the catalytic cycle [39]. Besides membrane physico-chemical properties, the modifications of the membrane lipid composition can also directly modulate the specific lipid-proteins interactions [29].

The plasma membrane Na, K-ATPase is such an integral protein whose activity is affected by membrane remodeling [29]. Its α catalytic subunit comprises 10 transmembrane helices important for ion binding and three cytosolic domains mediating ATP binding, ATP hydrolyzing activity or carrying phosphorylation sites. The Na, K-ATPase belongs to the P-type ATPase family characterized by ATP-dependent cyclic transition between two different conformational states, E1 and E2, which binds three Na^+ and two K^+ , respectively. Through model systems using reconstitution of the Na, K-ATPase into liposomes of defined lipid composition, its pump activity has been shown for a long time to be modulated by the physical properties of the lipid bilayer [29]. Later, thanks to the development of membrane protein crystallization and biochemical studies, three pockets were resolved in the TM domain that specifically bind anionic lipids, polyunsaturated neutral phospholipids and saturated PC conferring, respectively, stabilizing, stimulating and inhibiting effects on the Na, K-ATPase activity [29,40,41]. Interestingly, a correlation between a decreased Na, K-ATPase activity and a higher saturation level of membrane phospholipids in erythrocytes has been already demonstrated in hypertension cases [42,43]. For all these reasons and because we showed a dramatic increase in PC saturation closely correlated to both a K^+ efflux and NLRP3 inflammasome activation in SFA-treated human macrophages, we turned to the Na, K-ATPase. As expected, we observed a reduction of the K^+ uptake by the Na, K-ATPase in SFA-treated human macrophages. Accordingly, we propose the following model : in SFA-treated human macrophages, strongly increased saturated PC, by reducing the membrane fluidity and/or by modulating the lipid bilayer thickness or even through direct inhibiting interactions disrupt the plasma membrane Na, K-ATPase activity, which leads to a K^+ efflux followed by NLRP3 inflammasome activation. The link between all these events is reinforced by the preventing effect of UFAs on each step. Since the lipidomic analysis was performed on total lipid extracts, we cannot exclude the possibility that ER or mitochondria membranes are also damaged by the SFA treatment leading to the disruption of sarco/endoplasmic reticulum Ca^{2+} ATPase

(SERCA)-mediated Ca^{2+} homeostasis or mitochondrial dysfunction, both events previously related to NLRP3 activation [8].

We also investigated the mechanism by which UFAs prevent SFAs-mediated NLRP3 activation. Oleate does not compete with stearate to enter the macrophages, does not mediate its inhibiting effect by increasing the mitochondrial stearate flux but redirects the stearate flux from polar lipids to TAG stored in LD, thereby preventing the saturation of PC, the Na, K-ATPase disruption, the K^+ efflux and NLRP3 inflammasome activation. Although the inhibition of the TAG-synthetic enzyme, Diacylglycerol O-Acyltransferase 1 (DGAT1), reduced the radiolabeled stearate incorporation in TAG and slightly increased its flux into polar lipids in the presence of oleate, we could not see any effect on the ability of stearate co-treatment to abrogate the IL-1 β secretion (data not shown). This lack of effect could be explained by an higher flux of oleate towards polar lipids in the presence of DGAT1 inhibitor, that could counteract PC saturation. Robblee et al. did not observe any impact of oleate co-treatment on palmitate flux into lipids maybe because they used five-fold more palmitate than oleate while we always treated macrophages with equimolar stearate and oleate concentrations.

Western diet (WD) is rich both in saturated fat and carbohydrates. These latter are readily converted into SFAs via *de novo* lipogenesis in liver and adipocytes and delivered throughout the body via lipoproteins or as free fatty acids [44]. The composition of membrane phospholipids was reported to be influenced by the fatty acid composition of the diet [45–47]. These observations combined with compelling evidence of the NLRP3 central role in WD-induced systemic inflammation highlight the relevance of this NLRP3 activation model based on membrane saturation.

FFAs concentrations are also increased in the plasma and insulin-target tissues of obese patients [48]. Both a decrease of membrane fluidity and an enrichment of lipid species known to render the membrane phase more ordered have been observed in several kinds of cells in patients with T2DM (red blood cells, leucocytes, platelets, cardiac myocytes, ileal enterocytes,...) [49–52]. At least two large longitudinal studies demonstrated a correlation between the SFAs levels in red blood cells membranes and the susceptibility to develop T2DM [53,54]. In addition to the Na, K-ATPase, several integral membrane proteins involved in inflammatory and metabolic pathways such as TLR4, SERCA, IRE1,...are also modulated by the disruption of the membrane homeostasis and relevant in the context of obesity [36]. The "membrane-centric view of T2DM » is reinforced by this study demonstrating that NLRP3, a

central effector in the development of obesity-related inflammation and insulin resistance, also senses membrane damage. This work leads to novel therapeutic avenues such as those targeting some enzymes of the glycerolipid pathway.

Acknowledgments

This work was supported by the belgian F.R.S.-FNRS and the Leon Fredericq fondation from ULiege, Belgium. S.L-P and J.P. are research associate and research director of the belgian F.R.S.-FNRS, respectively. M.A.G. has a PhD fellowship from Walloon Region. N.P is MD, PhD in Medical sciences, specialized in internal medicine, endocrino-diabetology, full professor at ULiege, Belgium. The authors thank the Cell and Tissue Imaging Core (CIC, Impulse Financing KU Leuven, 2004) for the use of two-photon excitation microscope, and Dr Sandra Ormenese and the GIGA Cell Imaging Platform and Flow Cytometry Platform for their help in image acquisition with confocal microscope and flow cytometer respectively. We are grateful to Jean-François Liégeois, research director of the belgian F.R.S.-FNRS and assistant professor at ULiege, for the use of liquid scintillation analyser and stearate uptake experiment setups, and Michel Frédéricich, senior researcher at F.R.S.-FNRS and senior lecturer at the pharmacy department of ULiege, for the use of his laboratory material to realise TLC.

Authors contribution

M.A.G performed experiments and analyzed data. M.A.G and S. L-P conceived, designed and wrote the manuscript. J.P., N.P and L.L. contributed to the conception and design of the study. J.D. and J.V.S. contributed to the acquisition, analysis and interpretation of lipidomic and Laurdan microscopy data. N.E., Y.H., C.L., G.H., and O.J. contributed to helpful technical discussion or assistance. Each author revised and approved the manuscript. The authors declare that there is no conflict of interest associated with this manuscript.

REFERENCES

- [1] R.H. Eckel, S.M. Grundy, P.Z. Zimmet, The metabolic syndrome, *Lancet Lond. Engl.* 365 (2005) 1415–1428. doi:10.1016/S0140-6736(05)66378-7.
- [2] A.H. Mokdad, E.S. Ford, B.A. Bowman, W.H. Dietz, F. Vinicor, V.S. Bales, J.S. Marks, Prevalence of Obesity, Diabetes, and Obesity-Related Health Risk Factors, 2001, *JAMA.* 289 (2003) 76–79. doi:10.1001/jama.289.1.76.
- [3] N. Esser, S. Legrand-Poels, J. Piette, A.J. Scheen, N. Paquot, Inflammation as a link between obesity, metabolic syndrome and type 2 diabetes, *Diabetes Res. Clin. Pract.* 105 (2014) 141–150. doi:10.1016/j.diabres.2014.04.006.
- [4] J. Jager, T. Grémeaux, M. Cormont, Y. Le Marchand-Brustel, J.-F. Tanti, Interleukin-1 β -induced insulin resistance in adipocytes through down-regulation of insulin receptor substrate-1 expression, *Endocrinology.* 148 (2007) 241–251. doi:10.1210/en.2006-0692.
- [5] K. Maedler, P. Sergeev, F. Ris, J. Oberholzer, H.I. Joller-Jemelka, G.A. Spinas, N. Kaiser, P.A. Halban, M.Y. Donath, Glucose-induced beta cell production of IL-1 β contributes to glucotoxicity in human pancreatic islets, *J. Clin. Invest.* 110 (2002) 851–860. doi:10.1172/JCI15318.
- [6] K. Schroder, J. Tschopp, The inflammasomes, *Cell.* 140 (2010) 821–832. doi:10.1016/j.cell.2010.01.040.
- [7] P. Broz, V.M. Dixit, Inflammasomes: mechanism of assembly, regulation and signalling, *Nat. Rev. Immunol.* 16 (2016) 407–420. doi:10.1038/nri.2016.58.
- [8] T. Horng, Calcium signaling and mitochondrial destabilization in the triggering of the NLRP3 inflammasome, *Trends Immunol.* 35 (2014) 253–261. doi:10.1016/j.it.2014.02.007.
- [9] M.Y. Donath, É. Dalmas, N.S. Sauter, M. Böni-Schnetzler, Inflammation in obesity and diabetes: islet dysfunction and therapeutic opportunity, *Cell Metab.* 17 (2013) 860–872. doi:10.1016/j.cmet.2013.05.001.
- [10] S. Legrand-Poels, N. Esser, L. L’homme, A. Scheen, N. Paquot, J. Piette, Free fatty acids as modulators of the NLRP3 inflammasome in obesity/type 2 diabetes, *Biochem. Pharmacol.* 92 (2014) 131–141. doi:10.1016/j.bcp.2014.08.013.
- [11] B. Vandanmagsar, Y.-H. Youm, A. Ravussin, J.E. Galgani, K. Stadler, R.L. Mynatt, E. Ravussin, J.M. Stephens, V.D. Dixit, The NALP3/NLRP3 Inflammasome Instigates Obesity-Induced Autoinflammation and Insulin Resistance, *Nat. Med.* 17 (2011) 179–188. doi:10.1038/nm.2279.
- [12] H. Wen, D. Gris, Y. Lei, S. Jha, L. Zhang, M.T. Huang, W.J. Brickey, J.P. Ting, Fatty acid-induced NLRP3-ASC inflammasome activation interferes with insulin signaling, *Nat Immunol.* 12 (2011) 408–15. doi:10.1038/ni.2022.
- [13] N. Esser, L. L’homme, A.D. Roover, L. Kohnen, A.J. Scheen, M. Moutschen, J. Piette, S. Legrand-Poels, N. Paquot, Obesity phenotype is related to NLRP3 inflammasome activity and immunological profile of visceral adipose tissue, *Diabetologia.* 56 (2013) 2487–2497. doi:10.1007/s00125-013-3023-9.
- [14] H. Guo, J.B. Callaway, J.P.-Y. Ting, Inflammasomes: mechanism of action, role in disease, and therapeutics, *Nat. Med.* 21 (2015) 677–687. doi:10.1038/nm.3893.
- [15] R. Muñoz-Planillo, P. Kuffa, G. Martínez-Colón, B.L. Smith, T.M. Rajendiran, G. Núñez, K⁺ efflux is the Common Trigger of NLRP3 inflammasome Activation by Bacterial Toxins and Particulate Matter, *Immunity.* 38 (2013) 1142–1153. doi:10.1016/j.immuni.2013.05.016.
- [16] L. Franchi, T. Eigenbrod, R. Muñoz-Planillo, U. Ozkurede, Y.-G. Kim, A. Chakrabarti, M. Gale, R.H. Silverman, M. Colonna, S. Akira, G. Núñez, Cytosolic Double-Stranded RNA Activates the NLRP3 Inflammasome via MAVS-Induced Membrane Permeabilization and K⁺ Efflux, *J. Immunol.* 193 (2014) 4214–4222. doi:10.4049/jimmunol.1400582.
- [17] T. Próchnicki, M.S. Mangan, E. Latz, Recent insights into the molecular mechanisms of the NLRP3 inflammasome activation, *F1000Research.* 5 (2016). doi:10.12688/f1000research.8614.1.

- [18] Y. He, M.Y. Zeng, D. Yang, B. Motro, G. Núñez, Nek7 is an essential mediator of NLRP3 activation downstream of potassium efflux, *Nature*. 530 (2016) 354–357. doi:10.1038/nature16959.
- [19] M.M. Robblee, C.C. Kim, J.P. Abate, M. Valdearcos, K.L.M. Sandlund, M.K. Shenoy, R. Volmer, T. Iwawaki, S.K. Koliwad, Saturated Fatty Acids Engage an IRE1 α -Dependent Pathway to Activate the NLRP3 Inflammasome in Myeloid Cells, *Cell Rep.* 14 (2016) 2611–2623. doi:10.1016/j.celrep.2016.02.053.
- [20] L. L'homme, N. Esser, L. Riva, A. Scheen, N. Paquot, J. Piette, S. Legrand-Poels, Unsaturated fatty acids prevent activation of NLRP3 inflammasome in human monocytes/macrophages, *J. Lipid Res.* 54 (2013) 2998–3008. doi:10.1194/jlr.M037861.
- [21] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, *Methods San Diego Calif.* 25 (2001) 402–408. doi:10.1006/meth.2001.1262.
- [22] H.J. Dranse, M.E.M. Kelly, B.D. Hudson, Drugs or diet?--Developing novel therapeutic strategies targeting the free fatty acid family of GPCRs, *Br. J. Pharmacol.* 170 (2013) 696–711. doi:10.1111/bph.12327.
- [23] P.C. Calder, Long chain fatty acids and gene expression in inflammation and immunity, *Curr. Opin. Clin. Nutr. Metab. Care.* 16 (2013) 425–433. doi:10.1097/MCO.0b013e3283620616.
- [24] T.J. Grevenkoed, E.L. Klett, R.A. Coleman, Acyl-CoA metabolism and partitioning, *Annu. Rev. Nutr.* 34 (2014) 1–30. doi:10.1146/annurev-nutr-071813-105541.
- [25] M.C.E. Bragt, H.E. Popeijus, Peroxisome proliferator-activated receptors and the metabolic syndrome, *Physiol. Behav.* 94 (2008) 187–197. doi:10.1016/j.physbeh.2007.11.053.
- [26] C. Henique, A. Mansouri, G. Fumey, V. Lenoir, J. Girard, F. Bouillaud, C. Prip-Buus, I. Cohen, Increased Mitochondrial Fatty Acid Oxidation Is Sufficient to Protect Skeletal Muscle Cells from Palmitate-induced Apoptosis, *J. Biol. Chem.* 285 (2010) 36818–36827. doi:10.1074/jbc.M110.170431.
- [27] A.G. Jay, J.A. Hamilton, Disorder Amidst Membrane Order: Standardizing Laurdan Generalized Polarization and Membrane Fluidity Terms, *J. Fluoresc.* 27 (2017) 243–249. doi:10.1007/s10895-016-1951-8.
- [28] S. Mazeres, F. Fereidouni, E. Joly, Using spectral decomposition of the signals from laurdan-derived probes to evaluate the physical state of membranes in live cells, *F1000Research*. 6 (2017) 763. doi:10.12688/f1000research.11577.2.
- [29] F. Cornelius, M. Habeck, R. Kanai, C. Toyoshima, S.J.D. Karlish, General and specific lipid-protein interactions in Na,K-ATPase, *Biochim. Biophys. Acta.* 1848 (2015) 1729–1743. doi:10.1016/j.bbamem.2015.03.012.
- [30] A. Christ, P. Günther, M.A.R. Lauterbach, P. Duewell, D. Biswas, K. Pelka, C.J. Scholz, M. Oosting, K. Haendler, K. Baßler, K. Klee, J. Schulte-Schrepping, T. Ulas, S.J.C.F.M. Moorlag, V. Kumar, M.H. Park, L.A.B. Joosten, L.A. Groh, N.P. Riksen, T. Espevik, A. Schlitzer, Y. Li, M.L. Fitzgerald, M.G. Netea, J.L. Schultze, E. Latz, Western Diet Triggers NLRP3-Dependent Innate Immune Reprogramming, *Cell.* 172 (2018) 162–175.e14. doi:10.1016/j.cell.2017.12.013.
- [31] T. Karasawa, A. Kawashima, F. Usui-Kawanishi, S. Watanabe, H. Kimura, R. Kamata, K. Shirasuna, Y. Koyama, A. Sato-Tomita, T. Matsuzaka, H. Tomoda, S.-Y. Park, N. Shibayama, H. Shimano, T. Kasahara, M. Takahashi, Saturated Fatty Acids Undergo Intracellular Crystallization and Activate the NLRP3 Inflammasome in Macrophages, *Arterioscler. Thromb. Vasc. Biol.* 38 (2018) 744–756. doi:10.1161/ATVBAHA.117.310581.
- [32] K. Halbleib, K. Pesek, R. Covino, H.F. Hofbauer, D. Wunnicke, I. Hänelt, G. Hummer, R. Ernst, Activation of the Unfolded Protein Response by Lipid Bilayer Stress, *Mol. Cell.* 67 (2017) 673–684.e8. doi:10.1016/j.molcel.2017.06.012.
- [33] L.A.J. O'Neill, R.J. Kishton, J. Rathmell, A guide to immunometabolism for immunologists, *Nat. Rev. Immunol.* 16 (2016) 553–565. doi:10.1038/nri.2016.70.
- [34] D. Namgaladze, S. Lips, T.J. Leiker, R.C. Murphy, K. Ekroos, N. Ferreiros, G. Geisslinger, B. Brüne, Inhibition of macrophage fatty acid β -oxidation exacerbates palmitate-induced inflammatory

- and endoplasmic reticulum stress responses, *Diabetologia*. 57 (2014) 1067–1077. doi:10.1007/s00125-014-3173-4.
- [35] E. Rysman, K. Brusselmans, K. Scheys, L. Timmermans, R. Derua, S. Munck, P.P. Van Veldhoven, D. Waltregny, V.W. Daniëls, J. Machiels, F. Vanderhoydonc, K. Smans, E. Waelkens, G. Verhoeven, J.V. Swinnen, De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation, *Cancer Res.* 70 (2010) 8117–8126. doi:10.1158/0008-5472.CAN-09-3871.
- [36] M.A. Gianfrancesco, N. Paquot, J. Piette, S. Legrand-Poels, Lipid bilayer stress in obesity-linked inflammatory and metabolic disorders, *Biochem. Pharmacol.* (2018). doi:10.1016/j.bcp.2018.02.022.
- [37] H.-J. Kaiser, D. Lingwood, I. Levental, J.L. Sampaio, L. Kalvodova, L. Rajendran, K. Simons, Order of lipid phases in model and plasma membranes, *Proc. Natl. Acad. Sci. U. S. A.* 106 (2009) 16645–16650. doi:10.1073/pnas.0908987106.
- [38] M.D. Jensen, Fate of fatty acids at rest and during exercise: regulatory mechanisms, *Acta Physiol. Scand.* 178 (2003) 385–390. doi:10.1046/j.1365-201X.2003.01167.x.
- [39] Y. Li, M. Ge, L. Ciani, G. Kuriakose, E.J. Westover, M. Dura, D.F. Covey, J.H. Freed, F.R. Maxfield, J. Lytton, I. Tabas, Enrichment of endoplasmic reticulum with cholesterol inhibits sarcoplasmic-endoplasmic reticulum calcium ATPase-2b activity in parallel with increased order of membrane lipids: implications for depletion of endoplasmic reticulum calcium stores and apoptosis in cholesterol-loaded macrophages, *J. Biol. Chem.* 279 (2004) 37030–37039. doi:10.1074/jbc.M405195200.
- [40] M. Habeck, H. Haviv, A. Katz, E. Kapri-Pardes, S. Ayciriex, A. Shevchenko, H. Ogawa, C. Toyoshima, S.J.D. Karlsh, Stimulation, inhibition, or stabilization of Na,K-ATPase caused by specific lipid interactions at distinct sites, *J. Biol. Chem.* 290 (2015) 4829–4842. doi:10.1074/jbc.M114.611384.
- [41] M. Habeck, E. Kapri-Pardes, M. Sharon, S.J.D. Karlsh, Specific phospholipid binding to Na,K-ATPase at two distinct sites, *Proc. Natl. Acad. Sci. U. S. A.* 114 (2017) 2904–2909. doi:10.1073/pnas.1620799114.
- [42] R. Rodrigo, J.P. Bächler, J. Araya, H. Prat, W. Passalacqua, Relationship between (Na + K)-ATPase activity, lipid peroxidation and fatty acid profile in erythrocytes of hypertensive and normotensive subjects, *Mol. Cell. Biochem.* 303 (2007) 73–81. doi:10.1007/s11010-007-9457-y.
- [43] R. Rodrigo, A. Miranda-Merchak, R. Valenzuela Grau, J.P. Bachler, L. Vergara, Modulation of (Na,K)-ATPase activity by membrane fatty acid composition: therapeutic implications in human hypertension, *Clin. Exp. Hypertens. N. Y. N* 1993. 36 (2014) 17–26. doi:10.3109/10641963.2013.783048.
- [44] S. Kersten, Mechanisms of nutritional and hormonal regulation of lipogenesis, *EMBO Rep.* 2 (2001) 282–286. doi:10.1093/embo-reports/kve071.
- [45] S.K. Abbott, P.L. Else, T.A. Atkins, A.J. Hulbert, Fatty acid composition of membrane bilayers: Importance of diet polyunsaturated fat balance, *Biochim. Biophys. Acta BBA - Biomembr.* 1818 (2012) 1309–1317. doi:10.1016/j.bbamem.2012.01.011.
- [46] E.K. Lund, L.J. Harvey, S. Ladha, D.C. Clark, I.T. Johnson, Effects of dietary fish oil supplementation on the phospholipid composition and fluidity of cell membranes from human volunteers, *Ann. Nutr. Metab.* 43 (1999) 290–300.
- [47] A. Andersson, C. Nälsén, S. Tengblad, B. Vessby, Fatty acid composition of skeletal muscle reflects dietary fat composition in humans, *Am. J. Clin. Nutr.* 76 (2002) 1222–1229.
- [48] F. Soriguer, S. García-Serrano, J.M. García-Almeida, L. Garrido-Sánchez, J. García-Arnés, F.J. Tinahones, I. Cardona, J. Rivas-Marín, J.L. Gallego-Perales, E. García-Fuentes, Changes in the serum composition of free-fatty acids during an intravenous glucose tolerance test, *Obes. Silver Spring Md.* 17 (2009) 10–15. doi:10.1038/oby.2008.475.
- [49] M. Pilon, Revisiting the membrane-centric view of diabetes, *Lipids Health Dis.* 15 (2016). doi:10.1186/s12944-016-0342-0.

- [50] M. Borkman, L.H. Storlien, D.A. Pan, A.B. Jenkins, D.J. Chisholm, L.V. Campbell, The relation between insulin sensitivity and the fatty-acid composition of skeletal-muscle phospholipids, *N. Engl. J. Med.* 328 (1993) 238–244. doi:10.1056/NEJM199301283280404.
- [51] P.M. Clifton, P.J. Nestel, Relationship between plasma insulin and erythrocyte fatty acid composition, *Prostaglandins Leukot. Essent. Fatty Acids*. 59 (1998) 191–194.
- [52] E. Bakan, A. Yildirim, N. Kurtul, M.F. Polat, H. Dursun, K. Cayir, Effects of type 2 diabetes mellitus on plasma fatty acid composition and cholesterol content of erythrocyte and leukocyte membranes, *Acta Diabetol.* 43 (2006) 109–113. doi:10.1007/s00592-007-0224-4.
- [53] N.G. Forouhi, A. Koulman, S.J. Sharp, F. Imamura, J. Kröger, M.B. Schulze, F.L. Crowe, J.M. Huerta, M. Guevara, J.W.J. Beulens, G.J. van Woudenberg, L. Wang, K. Summerhill, J.L. Griffin, E.J.M. Feskens, P. Amiano, H. Boeing, F. Clavel-Chapelon, L. Dartois, G. Fagherazzi, P.W. Franks, C. Gonzalez, M.U. Jakobsen, R. Kaaks, T.J. Key, K.-T. Khaw, T. Kühn, A. Mattiello, P.M. Nilsson, K. Overvad, V. Pala, D. Palli, J.R. Quirós, O. Rolandsson, N. Roswall, C. Sacerdote, M.-J. Sánchez, N. Slimani, A.M.W. Spijkerman, A. Tjonneland, M.-J. Tormo, R. Tumino, D.L. van der A, Y.T. van der Schouw, C. Langenberg, E. Riboli, N.J. Wareham, Differences in the prospective association between individual plasma phospholipid saturated fatty acids and incident type 2 diabetes: the EPIC-InterAct case-cohort study, *Lancet Diabetes Endocrinol.* 2 (2014) 810–818. doi:10.1016/S2213-8587(14)70146-9.
- [54] J. Kröger, S. Jacobs, E.H.J.M. Jansen, A. Fritsche, H. Boeing, M.B. Schulze, Erythrocyte membrane fatty acid fluidity and risk of type 2 diabetes in the EPIC-Potsdam study, *Diabetologia*. 58 (2015) 282–289. doi:10.1007/s00125-014-3421-7.

Figure captions

Figure 1. ROS are not produced in human macrophages after FFAs treatments. (A) PMA-differentiated THP-1 cells were treated with BSA, FFAs (200 μ M) or antimycin A (50 μ M) for the indicated times followed by 30 min of incubation with MitoSOX (5 μ M) (top) or incubated with H2DCFDA (5 μ M) for 30 min followed by BSA, FFAs (200 μ M) or H₂O₂ treatment (1 mM) (bottom). Fluorescence was measured through EnSpire 2300 Multilabel Reader. (B) MDMs were pre-incubated with MitoSOX (5 μ M, 30min) at 37°C before being washed in warm culture medium and treated with BSA, FFAs (200 μ M, 6h) or antimycin A (50 μ M, 30min). At the end of incubation period, cells were subjected to flow cytometric analysis (BD Canto II) using the Phycoerythrin (PE) channel. A representative experiment is presented as fluorescence (% of Max) for the indicated treatments. Data were also quantified as fold change of median fluorescence intensity (MFI) compared with baseline and results are presented as means $\pm$ SEM of three independent experiments for FFAs.

Figure 2. IRE1 α endoribonuclease activity is not required for C18:0-mediated NLRP3 inflammasome activation in human macrophages. Effects of IRE1 α inhibitors (4 μ 8C and STF-083010) on spliced *Xbp1* mRNA expression (A, B) or IL-1 β secretion (C, D) after C18:0 treatment. Effects of phosphorylated eIF2 α inhibitor (ISRIB) on IL-1 β secretion (E, F) and ATF4 expression (G) in response to C18:0. PMA-differentiated THP-1 cells or MDMs were pre-treated for 1h with vehicle (DMSO) or with 4 μ 8C (50 μ M), STF-083010 (STF, 100 μ M) or ISRIB (10 nM) before addition of BSA (vehicle) or C18:0 (200 μ M) for 8h (THP-1+PMA) or 16h (MDMs). IL-1 β was measured in supernatants by ELISA and relative spliced *Xbp1* mRNA expression was assessed by qRT-PCR. HSP90 and ATF-4 were detected by immunoblotting in PMA-differentiated THP-1 cells extracts. Results are presented as means $\pm$ SEM of three (THP-1+PMA) and four (MDMs) independent experiments. *P < 0.05; ns, non significant by t-test.

Figure 3. SFAs activation into acyl-CoA is required for NLRP3 inflammasome activation but the β -oxidation is dispensable for both the SFA- and UFA-mediated effects. (A) SFAs activation into acyl-CoA is required for NLRP3 activation. (B, C) The SFAs flux in mitochondria is dispensable for both SFA-induced NLRP3 activation and UFA-mediated NLRP3 inhibition. PMA-differentiated THP-1 cells were pre-treated or not for 1h with triascin C (TC, 5 μ M) (A) or etomoxir (Eto, 25 μ M) (B) before stimulation for 8h with BSA, C16:0 (200 μ M), C18:0 (200 μ M)

alone or in combination with C18:1 (200 μ M+200 μ M) and Alum (400 μ g/ml) or with increasing Eto concentrations (1, 10, 50 and 100 μ M) (C) before stimulation with BSA or C16:0 (200 μ M) for 8h. IL-1 β release in supernatant (SN) and the Pro-IL-1 β , HSP60 or HSP90 levels in cytosol (XT) were determined by immunoblotting. A representative blot from minimal three independent experiments is shown.

Figure 4. Analysis of the C18:0 flux in the presence or not of C18:1. (A) C18:1 does not affect C18:0 uptake. PMA-differentiated THP-1 cells were treated with a mixture of 0,1 μ Ci/ml radiolabeled [1-14C] C18:0 and C18:0 (200 μ M) in the presence or not of C18:1 (200 μ M) during indicated times. Radioactivity in cell extracts was counted with liquid scintillation analyser to evaluate the radiolabeled [1-14C] C18:0 uptake. (B) The C18:0 flux is mainly channelled towards polar lipids and redirected towards TAG in the presence of C18:1. PMA-differentiated THP-1 cells were treated with a mixture of 2 μ Ci/ml radiolabeled [1-14C] C18:0 and C18:0 (200 μ M) in the presence or not of C18:1 (200 μ M) during 8 hours. Then total lipids were extracted and separated by TLC. Storage phosphor screens were exposed to the radioactive TLC for 4 hours and scanned with Typhoon 9400TM. Resulting digital image was quantified. Results are presented as means $\pm$ SEM of three independent experiments. *P < 0.05; **P < 0.01; ns, non significant by t-test. (C) C18:1 induces LD formation. PMA-differentiated THP-1 cells were either treated with vehicle (BSA), with C18:0 (200 μ M) or C18:1 (200 μ M) alone or with the combination C18:0+C18:1 (200 μ M+200 μ M) during 8 hours before being used for LD and nucleic acid staining with LipidTOX and DAPI, respectively. CE, cholesterol ester ; TAG, triacylglycerol ; FFA, free fatty acid ; 1,3-DAG, 1,3-diacylglycerol ; 1,2-DAG, 1,2-diacylglycerol ; PL, polar lipids.

Figure 5. C18:0 induces a dramatic increase in saturated PC that is prevented by C18:1 co-treatment. PMA-differentiated THP-1 cells were treated with BSA (vehicle), C18:0 (200 μ M) or C18:1 (200 μ M) alone or in combination C18:0+C18:1 (200 μ M+200 μ M) for 8h. Lipid extracts were used for lipidomic analysis. (A) The percentages of saturated (double bond=0), mono-unsaturated (double bond=1) and poly-unsaturated (double bond>1) species for each phospholipids class (PC, PE, PI, PS) were calculated for each treatment. Results are presented as means $\pm$ SEM of three independent experiments. Percentages of saturated species were compared between treatments for each phospholipid class : *P < 0.05; **P < 0.01; ***P < 0.001; ns, non significant by t-test. (B) Heatmap of log₂ ratios of the abundance of

phospholipids species in FFAs-treated PMA-differentiated THP-1 cells over vehicle (BSA)-treated cells. Log₂ fold change was arbitrarily set at -3.00/ 4.00 for colouring. Lipid species are indicated by their total number of fatty acid carbons, followed by the total number of unsaturations.

Figure 6. A strong decrease in membrane fluidity is induced by C18:0 but prevented by C18:1 co-treatment. PMA-differentiated THP-1 cells were either treated with vehicle (BSA), with C18:0 (200 µM) and with C18:1 (200 µM) alone or in combination C18:0+C18:1 (200 µM+200 µM) during 8 hours. After treatment, living cells were labeled with Laurdan (2,5 µM) for 1 hour and two-photon excitation microscopy was performed at room temperature. GP images of living cells were generated using the images acquired simultaneously in the two Laurdan emission spectra applying the GP formula and using imageJ. The colors indicate different GP values according to the reported scale going from 1 (gel phase) to -1 (fluid phase).

Figure 7. C18:0 induces a K⁺ efflux that is essential and closely correlated with IL-1β release.

(A,B) The K⁺ efflux is an essential step. **(A)** PMA-differentiated THP-1 cells were treated with Alum (400 µg/ml) in presence of increasing extracellular [K⁺]. **(B)** Both PMA-differentiated THP-1 cells and MDMs were stimulated with C18:0 (200 µM) in presence of increasing extracellular [K⁺] during 8 or 16 hours, respectively. Supernatants (SNs) were harvested for IL-1β measurements by ELISA. The levels of Pro-IL-1β and HSP90 in cytosol (XT) were determined by immunoblotting. Results are presented as means ± SEM of three (PMA-differentiated THP-1 cells) or four (MDMs) independent experiments. **P < 0.01; ***P < 0.001; ****P < 0.0001 by t-test. **(C,D)** The K⁺ efflux is tightly correlated to IL-1β secretion. **(C)** PMA-differentiated THP-1 cells were either treated with vehicle (DMSO), Alum (400 µg/ml), Nigericin (10µM) or Ouabain (1 µM) or **(D)** with BSA, C18:0 (200 µM) or C18:1 (200µM) alone or in combination C18:0+C18:1 (200 µM+200µM) during indicated times. SNs were harvested for IL-1β measurements by ELISA and cell lysates in 10% nitric acid were analyzed by atomic emission spectroscopy to determine intracellular [K⁺]. Results are presented as means ± SEM of three independent experiments. **P < 0.01; ***P < 0.001 by t-test.

Fig.8. The activity of the plasma membrane Na, K-ATPase is down-regulated by SFAs treatment. **(A)** PMA-differentiated THP-1 cells were either treated with vehicle (BSA), with C16:0 (200 µM), C18:0 (200 µM), C18:1 (200 µM) or with the combination C18:0+C18:1 (200

μM +200 μM) during 8 hours. **(B)** MDMs were either treated with vehicle (BSA), C16:0 (400 μM) or C18:0 (400 μM) during 8 hours. The Na,K-ATPase activity was estimated by ouabain-sensitive $^{86}\text{Rb}^+$ uptake. The levels of the Na,K-ATPase $\alpha 1$ subunit and α -Tubulin were determined by immunoblotting on membrane (M) and cytoplasmic (C) cell fractions, respectively. Results are presented as means $\pm$ SEM of five (PMA-differentiated THP-1 cells) or four (MDMs) independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; ns, non significant by t-test.

Figure 1

Figure 2

Figure 3

Figure 4

A

Figure 6

Figure 7

Figure 8

Graphical abstract