Immunometabolism in osteoarthritis

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Chapter 6

The fate of oleic acid in CD4$^+$ T cells

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Abstract

The metabolism of immune cells is intimately linked to their function. We have previously shown that exposure to fatty acids enhances proliferation of CD4+ T cells. In this study, we analysed the contribution and fate of oleic acid (OA) to T cell proliferation to get insight into the underlying mechanisms.

Purified CD4+ T cells from healthy volunteers were activated by αCD3/CD28 in the presence of 0.1, 1 or 10 μg/ml OA and proliferation was measured on day 4. Glycolysis and mitochondrial respiration of CD4+ T cells were assessed using Seahorse technology. Metabolomics studies were performed using 13C-oleic acid (13C-OA) and liquid chromatography/mass spectrometry (LC-MS). Moreover, 13C-containing lipid species were determined by gas chromatography or LC-MS. Furthermore, TCR signalling was assessed by measuring calcium flux and ZAP70 phosphorylation.

OA enhanced αCD3/CD28-induced proliferation, even when OA was removed after 24 hours, indicating that early OA-induced changes can prime T cells for enhanced proliferation. Functional metabolic analyses indicated an increase in glycolysis and in some donors in mitochondrial respiration upon activation of T cells, although both were unaffected by OA treatment. In line with these data, metabolomics analysis indicated an increase in overall metabolic parameters upon activation of T cells for 24 hours but only a modest effect of OA on glycolysis and TCA cycle intermediates. Similarly, inhibition of fatty acid transport into mitochondria using etomoxir did not affect OA-mediated enhanced proliferation, indicating that changes in cellular metabolism do not contribute substantially to the observed effects of OA. Interestingly, OA enhanced pyrimidine synthesis after 8 hours and 24 hours. By using 13C-OA we found OA is taken up by cells and incorporated predominantly into phosphatidylcholines. Furthermore, we found that OA enhanced Ca-flux responses without affecting phosphorylation of ZAP70.

Our study indicates that rather than used as energy source, OA is used as membrane building block influencing calcium signalling in the T cell which may allow for stronger cellular activation in response to TCR triggering.

Introduction

The metabolism of $CD4^+$ T cells and their function are intimately linked. Naïve resting $CD4^+$ T cells have a quiescent state and generally depend on the breakdown of glucose, fatty acids and amino acids to fuel oxidative phosphorylation (OXPHOS) to maintain their homeostasis [1]. However, in order to proliferate, $CD4^+$ T cells need to adjust their metabolism and enhance growth-promoting pathways. To this end, aerobic glycolysis is greatly enhanced, as well as OXPHOS [1, 2]. Aerobic glycolysis is fuelled by the enhanced uptake of glucose through the upregulation of glucose transporter 1 (GLUT1) and will generate ATP and lactate from glucose [1, 3, 4]. Although this pathway is relatively inefficient in producing ATP, it has the benefit of generating metabolic intermediates that can be used to produce macromolecules used for cell growth and proliferation, such as nucleic acids, amino acids or lipids. In addition, uptake of amino acids, such as glutamine is increased and can either directly serve as building block for protein synthesis or serve as fuel for the TCA cycle, which also will generate intermediates to produce macromolecules [2, 5-7].

Fatty acids have been shown to affect both proliferation and function of $CD4^+$ T cells [8]. However, little is known about the dependence of $CD4^+$ T cells on fatty acids for their proliferation. Fatty acid oxidation (FAO) has been shown to be crucial for the development of T regulatory (Treg) cells. In contrast, FAO suppresses T effector cells which are dependent on fatty acid synthesis [3, 9, 10]. Furthermore, FAO has been shown to be important for memory T cells, as FAO promotes effector memory $CD4^+$ T cells [11]. Moreover, $CD8^+$ T cells are reliant on FAO to persist in skin tissue [12]. Some studies suggested that exogenous fatty acids fuel FAO [9, 12], however, recently it has been shown that $CD8^+$ memory T cells use extracellular glucose and endogenous lipids to support FAO rather than using exogenous fatty acids directly [13, 14]. In addition, it has been shown that both fatty acid biosynthesis and uptake are needed for activation and proliferation of $CD4^+$ T cells [15]. Overall, the exact mechanism by which exogenous fatty acids contribute to FAO and influence $CD4^+$ T cells is poorly understood.

In obese persons the levels of free fatty acids in plasma are elevated [16, 17]. The effect of free fatty acids on T cells appears to be concentration-dependent: low concentrations leads to enhanced proliferation, while high concentrations lead to apoptosis [8]. The underlying mechanisms leading to enhanced proliferation in the

presence of exogenous fatty acids are yet unknown. Possible mechanisms include oxidation of fatty acids to generate energy or incorporation of fatty acids into complex macromolecules used as signalling molecules or building blocks for the daughter cells. The aim of this study was to gain more insight into the mechanisms underlying the enhanced proliferation of CD4+ T cells upon exogenous fatty acid uptake. To this end we used oleic acid (OA) as a model free fatty acid, as it was previously shown to enhance CD4+ T cell proliferation [18].

Materials and methods

Peripheral blood CD4+ T cell isolation

PBMCs were isolated from heparinized blood or buffy coats of healthy blood bank donors by standard ficoll plaque gradient. Purification for CD4+ T cells was performed using Dynabeads FlowComp Human CD4 isolation kit (Invitrogen Dynal, Oslo, Norway) according to manufacturer's protocol. In short, PBMCs were incubated with an anti-CD4 antibody which binds to the target cells. CD4+ T cells which have bound the specific antibodies were captured by magnetic beads followed by removal of magnetic beads. The purity of CD4+ T cells was typically above 95%. Written informed consent was obtained from all donors and the study was approved by the local medical ethical committee.

T cell proliferation assays

Purified CD4+ T cells were plated in a density of 100.000 cells/well in 200 µl in a 96-well plate in DMEM high glucose (4.5 g/l) with no sodium pyruvate (Sigma Aldrich, Germany), supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax (medium). Cells were activated with 5 µg/ml plate-bound (pb.) αCD3 (clone OKT3, eBioscience, San Diego, USA) and 1 µg/ml soluble (sol.) αCD28 (Sanquin, Amsterdam, The Netherlands). Cells were treated with 0.1, 1 or 10 µg/ml oleic acid (Sigma Aldrich, Germany) or 0.1, 1 or 10 µg/ml U-13C18 OA (U13C OA) (Cambridge Isotope Laboratories, United Kingdom) or ethanol control (EtOH) (solvent control). OA diluted in EtOH was conjugated to fatty acid free BSA by addition of DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax, followed by 2 rounds of vortexing and sonication (30 sec). For inhibition experiments, etomoxir (ETO, 50 µM, 100 µM or 200 µM), C75 (20 µM and

40 µM), TOFA (2 µM and 20 µM) or DMSO control were added to the cultures for the indicated periods of time. For short-term treatment, OA and inhibitors were removed after 24 hours by 3 rounds of washing and fresh medium with 1 µg/ml soluble αCD28 was added to the cells. Proliferation during the last 18-20 hours of a 4 day culture was measured upon addition 0.5 µCi/well ^{3}H-Thymidine.

CTV staining

$CD4^+$ T cells were labelled with 5 µM cell trace violet (CTV) (Life Technologies) in 1 ml of PBS/0.1%BSA at 37°C. After 15 minutes cells were washed three times with cold 10% FCS/medium after which cells were taken up in DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax and plated in a density of 100.000 cells/well in 200 µl medium in a 96-well plate. Cells were stimulated with 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 and treated with 0.1, 1 or 10 µg/ml OA or solvent control. After 24 hours cells were harvested and CTV dilution was measured on LSR-II (BD Bioscience) or OA was washed away and cells were harvested on day 4 to measure CTV dilution on LSR-II.

T cell metabolism assays

Purified blood $CD4^+$ T cells were plated in 50 µg/ml poly-D-lysine (Millipore) coated XF-plates (Seahorse) in a density of 300.000 cells/well in 160 µl XF media (nonbuffered DMEM high glucose (4.5 g/l) with no sodium pyruvate (Sigma Aldrich, Germany), supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax). Cells were incubated for 1 hours in a non-CO2 incubator. Prior to measuring of the oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) 40 µl of 0.1 µg/ml, 1 µg/ml or 10 µg/ml OA or solvent control was added. Basal OCR and ECAR levels were measured as well as levels after injection of 300.000 Dynabeads® Human T-Activator CD3/CD28 (Invitrogen) using an XF-96 Flux analyser (Seahorse Bioscience). In addition, unstimulated or 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 stimulated $CD4^+$ T cells were pre-treated for 24 hours with OA after which cells were transferred to poly-D-lysine coated XF-plates (Seahorse) and incubated for 1 hours in a non-CO2 incubator after which basal OCR and ECAR levels were measured.

T cell metabolomics

Unstimulated or 5 µg/ml pb. αCD3 and 1 µg/ml sol. αCD28 stimulated cells were plated in 200 µl DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a density of 300.000 cells/well in a 96-well plate and treated with 0.1, 1 or 10 µg/ml OA or 0.1, 1 or 10 µg/ml U13C OA. After 8 and 24 hours, 4 wells per condition were pooled and cells ($1.2x10^6$ cells) were washed with ice cold PBS and metabolites were extracted in 50 µl lysis buffer containing methanol/acetonitrile/dH2O (2:2:1). Samples of medium cultured for 8 and 24 hours were also collected. One ml of lysis buffer was added to 10 µl of medium to extract metabolites. Samples were spun at 16.000g for 15 minutes at 4 °C. Supernatants were collected for LC-MS analysis. LC-MS analysis was performed on an Exactive mass spectrometer (Thermo Scientific) coupled to a Dionex Ultimate 3000 autosampler and pump (Thermo Scientific). The MS operated in polarity-switching mode with spray voltages of 4.5 kV and -3.5 kV. Metabolites were separated using a Sequant ZIC-pHILIC column (2.1 x 150 mm, 5 µm, guard column 2.1 x 20 mm, 5 µm; Merck) using a linear gradient of acetonitrile and eluent A (20 mM (NH4)2CO3, 0.1% NH4OH in ULC/MS grade water (Biosolve)). Flow rate was set at 150 µl/min. Metabolites were identified and quantified using LCquan software (Thermo Scientific) on the basis of exact mass within 10 ppm and further validated by concordance with retention times of standards. Peak intensities were normalized based on total ion count (TIC).

Uptake of OA

Purified CD4+ T cells were treated as specified under T-cell metabolomics. After 24 hours, cells were washed with 180 µl of PBS (pH 7.4 [-] CaCl2 and [-] MgCl2) (Gibco). For GC-MS analysis cell pellet was resuspended in 20 µl PBS (pH 7.4 [-] CaCl2 and [-] MgCl2) and transferred into 250 µl acetone in an Agilent glass vial. Remaining cell pellet was resuspended in 10 µl EtOH and transferred to the same vial. Lipids were hydrolyzed at 60 ˚C for 30 min. using 10 µl 10 M sodium hydroxide if the total fatty acid content was planned to be analyzed. Thereafter 10 µl 1 µg/ml palmitic acid-d31 (Cambridge Isotope Laboratories) in ethanol was added and samples were homogenized. Fatty acids were derivatized by adding 100 µl 172 mM PFBBr in acetone and the reaction was allowed to proceed for 30 min. at 60 ˚C. Then a liquid-liquid extraction was performed using 500 µl n-hexane and 250 µl water, the upper, n-hexane, layer was transferred into an Agilent auto sampler vial with

glass insert and analyzed using GC-MS. For analysis of calibration range samples 10 µl U13C OA in EtOH and for medium samples 10 µl medium was used instead of 30 µl PBS/EtOH containing T-cells. Fatty acid content was analyzed using a Bruker Scion 436 GC coupled to Bruker Scion TQ MS. Injection was performed using an Agilent CTC PAL autosampler (G6501-CTC): 1 µl sample was injected splitless at 280 ˚C. The GC was equipped with an Agilent VF-5ms (25 m × 0.25 mm i.d.,0.25 µm film thickness) column, helium was used as carrier gas at a flow of 1.20 ml/min. The following temperature gradient was used to separate the compounds: 1 min. 50 ˚C, linear increase at 40 ˚C/min. to 60 ˚C, 3 min. at 60 ˚C, linear increase at 25 ˚C/min to 237 ˚C, linear increase at 3 ˚C/min to 250 ˚C, linear increase at 25 ˚C/min to 315 ˚C and 1.55 min. at 315 ˚C. The total analysis time per sample was 19.81 min. The transfer line and ionization source temperature were 280 ˚C. The pressure of the chemical ionization gas, methane, was set at 15 psi. Negatively charged ions were detected in the Selected Ion Monitoring mode. U13C OA was quantified using GC-MS data. An external calibration of U13C OA was used, palmitic acid-d31 served as internal standard for this calibration. The calibration curve was weighted using a weight factor of two on the concentration.

Incorporation of OA

Purified $CD4^+$ T cells were treated as specified under T -cell metabolomics. After 24 hours, cells were washed with 180 µl of PBS (pH 7.4 [-] CaCl2 and [-] MgCl2). For LC-FT/MS analysis, 3 wells were pooled and resuspended in 20 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and transferred into 295 µl of isopropanol in an 1.5 ml eppendorf tube, then the remainder of cells was resuspended in 10 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and transferred into the isopropanol. For calibration range samples 30 µl PBS (pH 7.4 [-] CaCl2, [-] MgCl2) and for medium samples 30 µl of medium was added to 290 µl (calibration range samples) or 295 µl (medium samples) isopropanol. Then 5 µl of an internal standard containing ergosterol acetate (gift from prof. Bracher), LPC (19:0) (Avanti Polar Lipids), PC (11:0/11:0) (Avanti Polar Lipids), PE (15:0/15:0) (Avanti Polar Lipids) and TG (17:0/17:0/17:0) (Sigma Aldrich) in isopropanol was added. The final concentration, the concentration to be analyzed on the LC-FT/MS, of these lipids was 0.1 µg/ml except for ergosterol acetate which final concentration was 1 µg/ml. The samples were sonicated for 5 min. and spun at 16.100g for 5 min. Finally, 100 µl of supernatant was complemented with 100 µl water and put into an Agilent auto sampler vial with glass insert. Samples were measured immediately or stored at -80 ˚C until analysis. Lipids were analysed using a Dionex Ultimate 3000

LC-system coupled to a 12T Bruker solariX XR mass spectrometer. 10 µl of sample was injected and separated on a phenomenex kinetex 1.7 µm, c18 (50 x 2.1 mm; 100 Å) column, which was held at 50 ˚C. The mobile phase flow rate was 250 µl /ml and a gradient of eluent A (80% water, 20% acetonitrile, 5 mM ammonium formate and 0.05% formic acid) and B (90% isopropanol, 9% acetonitrile, 1% water, 5 mM ammonium formate and 0.05% formic acid) was used to elute the lipids from the column. The gradient was programmed as follows: 0-1 min. 50% B, 1-8 min. linear increase to 95% B, 8-10 min. stable at 95% B, 10-10.5 min linear decrease to 50% B and 10.5-15 min stable at 50% B. A post column flow splitter was used. Compounds were ionized using electron spray ionization. N_2 was used as nebulizer (1.3 bar) and drying gas (4.0 L/min.), the dry temperature of the source was set at 200 ˚C. The capillary voltage was 4300 V. Compounds were detected in the positive mode, m/z-values between 200 and 1200 were detected. Masses in the spectra of LC-FT/MS data were internally calibrated using the monoisotopic mass of LPC (19:0), PC (11:0/11:0), PE (15:0/15:0) and TG (17:0/17:0/17:0). Extracted Ion Chromatograms of the compounds to be quantified were formed. PCs and TGs were quantified using an external calibration curve of PC (19:0/19:0) (Avanti Polar Lipids) and TG (15:0/15:0/15:0) (Sigma Aldrich), respectively. As internal standard for the quantitation of PCs and TGs, PC (11:0/11:0) and TG (17:0/17:0/17:0) were used respectively. Linear calibration curves were weighted using a weight factor of two on the concentration [19].

Ca flux assay

Purified CD4+ T cells were incubated with 10 µg/ml OA or EtOH in a density of $3x10^6$ cells/well in 2.5 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a 6-well plate. After 24 hours cells were harvested and $2x10^6$ were stained for 35 minutes at 37°C with 2µM Indo-1 AM (Thermo Scientific) in 0.2 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA in the presence of 0.02% pluronic acid and 5 µg/ml αCD3 and 1 µg/ml αCD28. Cells were washed and taken up in 1 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 2% fatty acid free BSA and 1 mM $CaCl_2$. Before measuring cells were warmed up to 37°C in a waterbath and baseline was measured on LSR-II for 2 minutes before addition of 3 µg/ml goat-anti-mouse antibody (Dako) to cross-link αCD3 and αCD28 after which calcium flux was measured for an additional 5 minutes.

pZAP-70 WB

Purified $CD4^+$ T cells were incubated with 10 µg/ml OA or EtOH in a density of $2.5x10^6$ cells/well in 2.5 ml DMEM high glucose (4.5 g/l) with no sodium pyruvate, supplemented with 0.5% fatty acid free BSA, 100 U/ml penicillin/streptomycin and 2 mM glutamax in a 6-well plate. Cells were harvested after 24 hours and $4x10^6$ cells were incubated with 25 µg/ml αCD3 and 5 µg/ml αCD28 or medium as control in 500 µl for 15 min at 37°C in tubes. Cells were washed and cross-linking of αCD3 and αCD28 was achieved by incubating cells with 5 µg/ml goat-anti-mouse antibody in 500 µl medium at 37°C for 30 sec, 2 min or 5 min. Stimulation of T cells by cross-linking was stopped by addition of 1 ml of ice-cold PBS. Cells were washed with ice-cold PBS and taken up in 30 µl NP-40 lysis buffer (Invitrogen) in presence of 1:100 phosphatase/protease inhibitors (Cell Signaling). Cells were lyzed on ice for at least 1 hours while vortexing every 15 min and centrifuged for 15 min at max speed. Supernatants were collected and stored until use. Protein content of cell lysates was measured using the BCA assay (Thermo Scientific). Ten µg protein was diluted 4x with Laemmli sample buffer (Bio-Rad)/5% β-mercaptoethanol (Merck) and boiled for 5 min at 95°C. Proteins were separated by SDS/PAGE on a mini-protean precast gel (Bio-Rad) followed by protein transfer on a Trans-Blot Turbo Transfer pack (Bio-Rad). The membrane was washed and blocked with TBS/0.1% Tween/5% skim milk powder (Fluka) for 1 hour at RT under continuous rolling. Next, the membrane was incubated with zeta chain of TCR associated protein kinase (Zap70) rabbit antibody or pZap70 (Y319) Rabbit antibody (all from Cell Signaling) overnight at 4°C. The membrane was washed and incubated with anti-Rabbit HRP antibody for 1 hours at room temperature after which the membrane was washed with TBS/0.1% Tween and milliQ water. Zap70 or phosph-Zap70 was visualized using ECL solution.

Results

Oleic acid enhances proliferation of $CD4^+$ T cells during the first 24 hours

Oleic acid (OA) was used as a model fatty acid to study the effects of free fatty acids on $CD4^+$ T cells. A dose-dependent response in proliferation was observed, 4 days after αCD3/CD28 stimulation, when OA was added to the cells (Fig 1A), even when OA was removed after 1 day (Fig 1B). To get more insight into the kinetics of the proliferative response, we have also measured T cell proliferation at 24 hours.

A dose-dependent increase in tritium Thymidine incorporation was observed in response to OA treatment also at this early timepoint (Fig 1C). Enhanced cell proliferation in the presence of OA was also observed using CTV staining (Fig 1E), although this was less evident on day 1 (Fig 1D). These data indicate that the presence of OA during the first 24 hours of T cell stimulation enhances cell division.

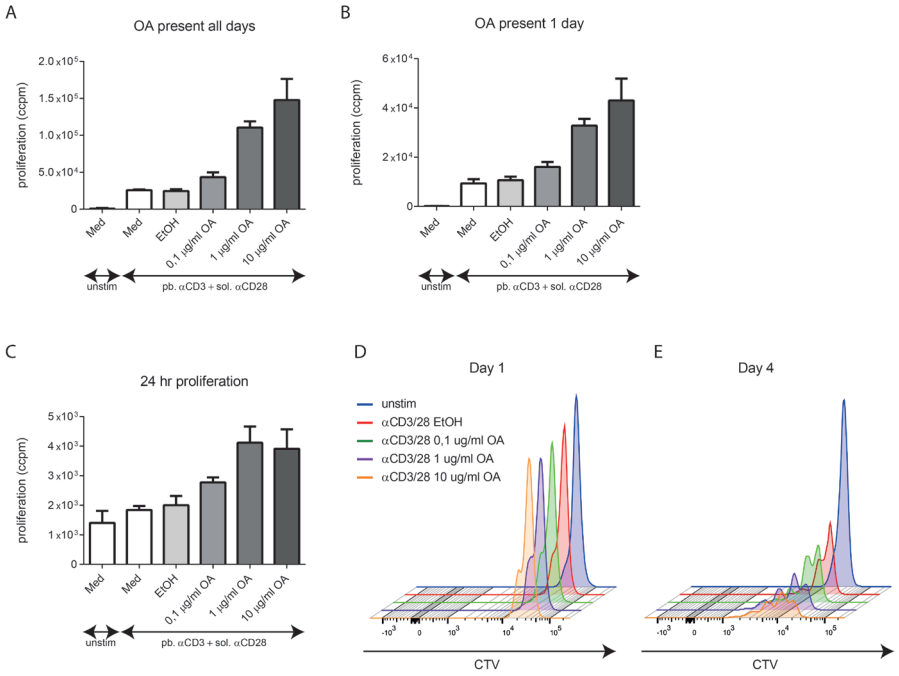

Figure 1. Oleic acid enhances proliferation of CD4+ T cells during the first 24 hours

Isolated CD4+ T cells were incubated with 0.1, 1 or 10 µg/ml OA or controls in the presence of pb. αCD3 and sol. αCD28. T cell proliferation was determined after 4 days with OA present the whole period of time (A) or OA washed away after 24 hours (B) or proliferation was determined after 24 hours of incubation with OA (C). Data are shown as mean + SD of triplicates and are from one representative experiments out of 3-6 performed. Isolated CD4+ T cells were labeled with CTV after which cells were incubated with 0.1, 1 or 10 µg/ml OA or controls in the presence of pb. αCD3 and sol. αCD28. After 24 hours (D) or after 4 days (E) dilution of CTV was determined. Representative plot out of 4 performed is shown.

Oleic acid does not affect glycolysis or mitochondrial respiration of CD4+ T cells

To study the effect of OA on CD4+ T cell metabolism, glycolysis and mitochondrial respiration was measured using Seahorse technology. Stimulation of CD4+ T cells enhanced glycolysis (Fig 2A), without affecting mitochondrial respiration (Fig 2C). Both metabolic pathways were unaffected by the OA treatment (Fig 2B and D). Stimulation of CD4+ T cells for 24 hours resulted in enhanced glycolysis (Fig 2E) and

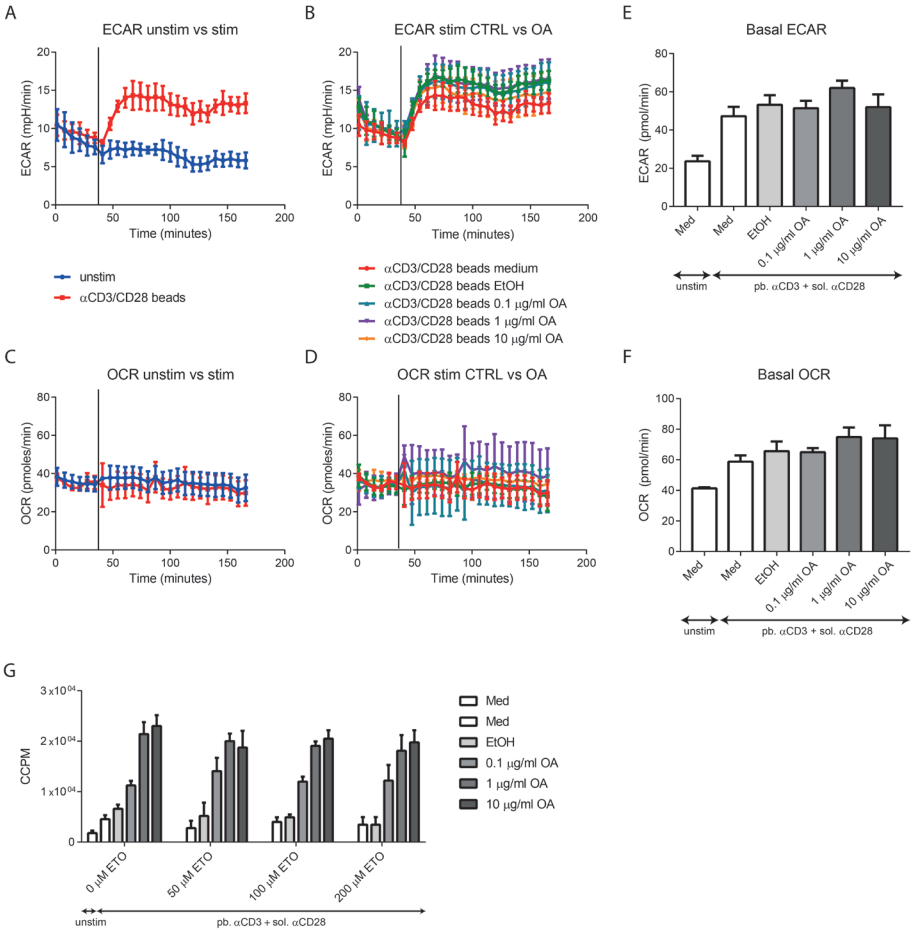

Figure 2. Oleic acid does not affect glycolysis or mitochondrial respiration of $CD4^+$ T cells

Isolated $CD4^+$ T cells were incubated with 0.1, 1, 10 μg/ml OA prior to measuring ECAR and OCR. ECAR (A and B) and OCR (C and D) was measured for 200 minutes upon activation with αCD3/αCD28 coated beads (indicated with line). Data are shown as mean + SD of quadruplicates and are from one representative experiments out of 6 performed. Basal ECAR (E) and OCR (F) of $CD4^+$ T cells treated with 0.1, 1, 10 μg/ml OA in presence of pb. αCD3 and sol. αCD28 for 24 hours was measured. Data are shown as mean + SD of quadruplicates and are from one representative experiments out of 8 performed. Proliferation of pb. αCD3 and sol. αCD28 stimulated $CD4^+$ T cells treated with 0.1, 1, 10 μg/ml OA in presence of 50 μM, 100 μM or 200 μM ETO or control for 24 hours after which OA and ETO was washed away and proliferation was measured on day 4. Data are shown as mean + SD of triplicates and are from one representative experiments out of 4 performed.

enhanced mitochondrial respiration, although the latter was donor-dependent (Fig 2F). Again, OA treatment did not affect any of these pathways (Fig 2E and F). In line with these findings, blocking of OA import into mitochondria using etomoxir did not influence the enhanced proliferation induced by OA (Fig 2G),

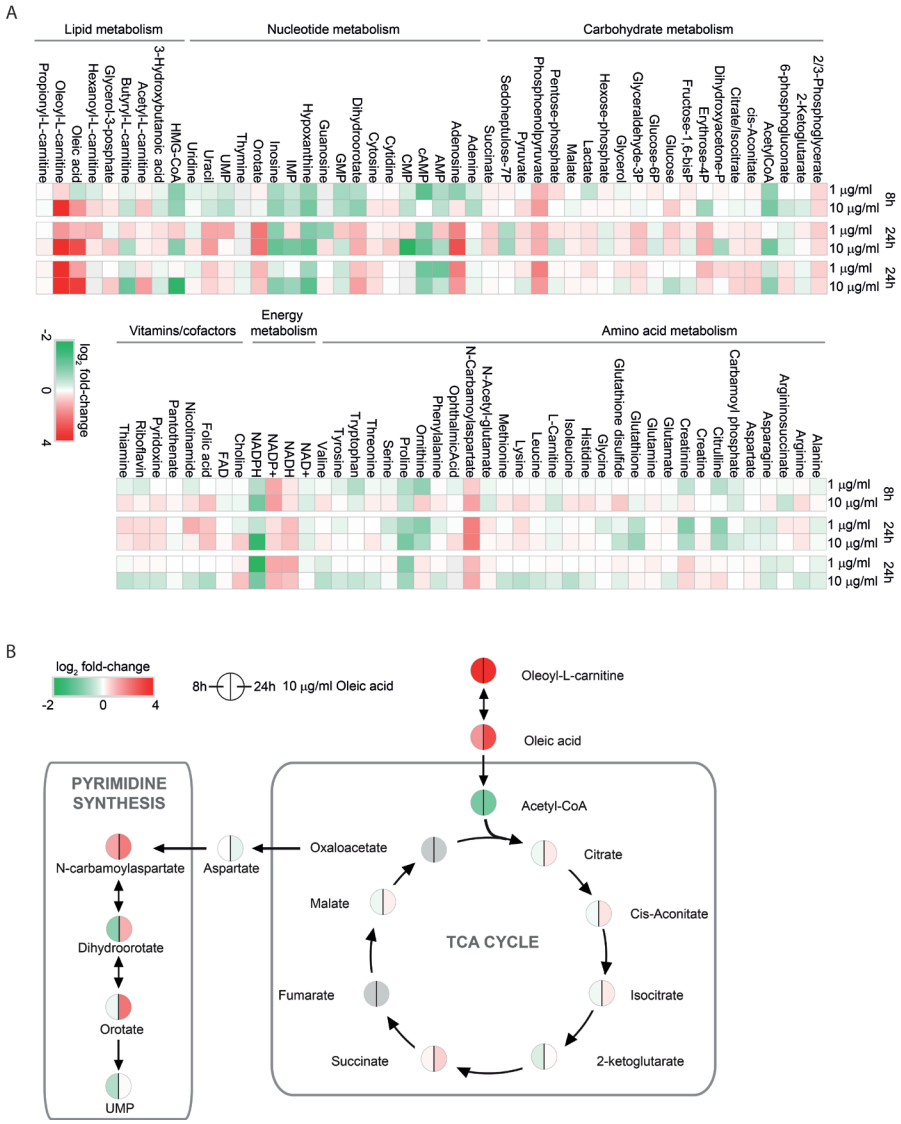

Figure 3. Oleic acid enhances TCA cycle and pyrimidine synthesis

T cells were incubated with 1 or 10 µg/ml OA in presence of pb. αCD3 and sol. αCD28 for 8 or 24 hours after which LC-MS analysis was performed and metabolites were determined. Metabolites decreased in OA treated samples compared to EtOH treated samples were displayed in green, while metabolites increased in OA treated samples compared to EtOH were displayed in red. Experiment was performed twice with time point 24 hours (A). TCA cycle and pyrimidine synthesis showed differences between EtOH and OA and is shown in detail (B).

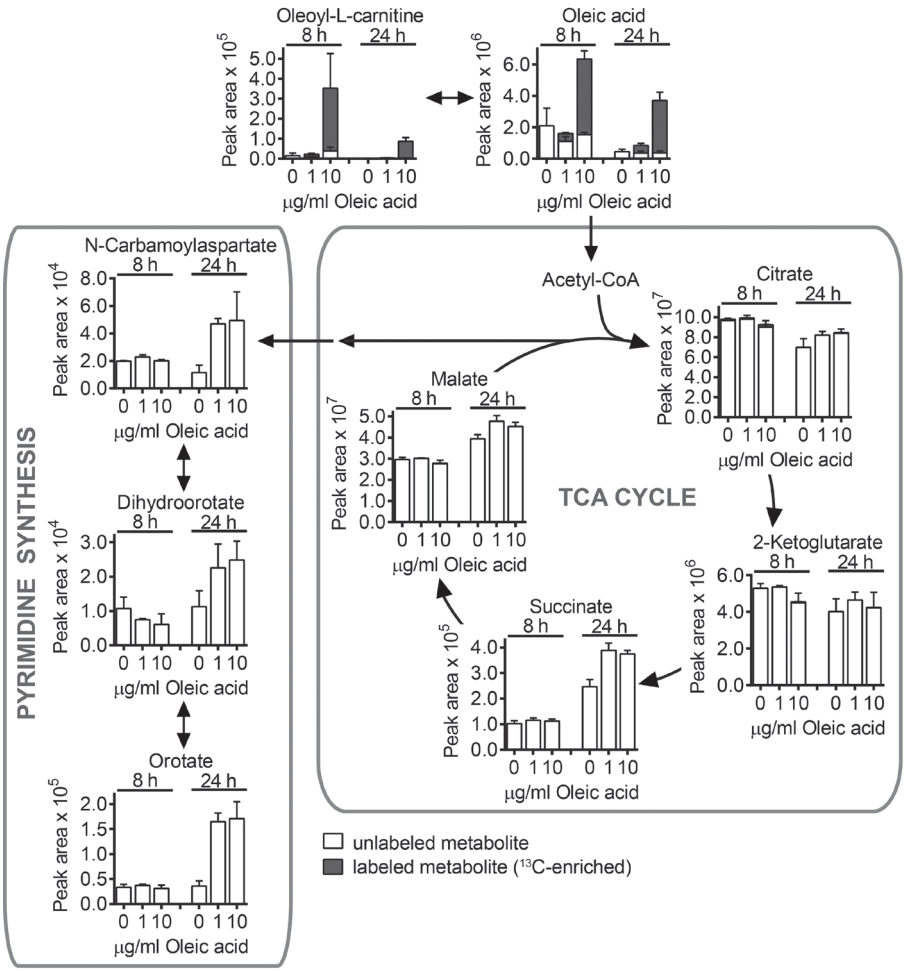

Figure 4. Oleic acid is not used as energetic substrate

CD4+ T cells were incubated with 1 or 10 µg/ml 13C-OA in presence of pb. αCD3 and sol. αCD28 for 8 or 24 hours after which LC-MS analysis was performed and unlabeled and labelled metabolites were determined. White bars represent unlabeled metabolites, grey bars represent labelled metabolites.

suggesting that OA treatment does not affect glycolysis and mitochondrial respiration.

Oleic acid enhances TCA cycle and pyrimidine synthesis

To determine whether OA does not affect any other metabolic pathway, a complete metabolomics analysis was performed after incubating CD4+ T cells for 8 hours or 24 hours in the presence of OA. These analyses demonstrated that stimulation

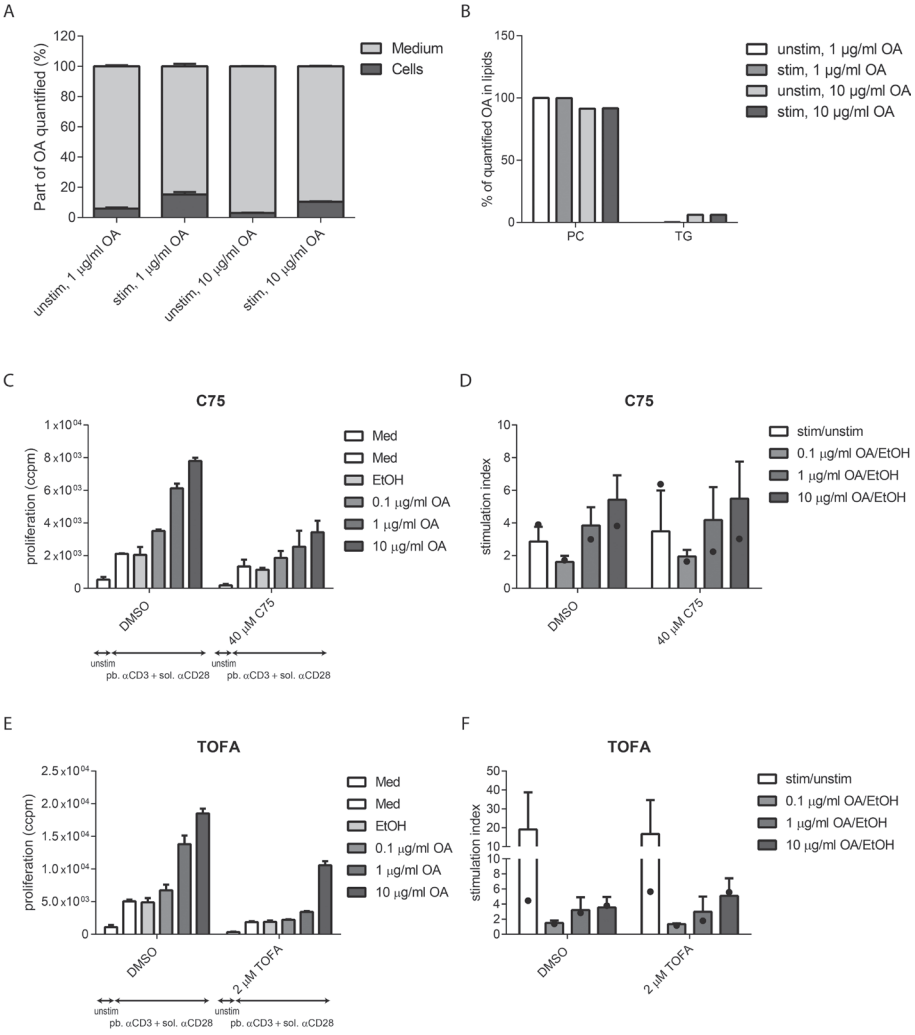

Figure 5. Oleic acid is incorporated in phosphatidylcholines

Isolated CD4+ T cells were treated with 1 or 10 µg/ml 13C-OA in presence or absence of pb. αCD3 and sol. αCD28 for 24 hours and GC-MS was used to determine the percentage of OA taken up by cells (A). LC-FT/MS was used to determine in which higher order lipids 13C-OA was incorporated (B). Proliferation of pb. αCD3 and sol. αCD28 stimulated CD4+ T cells treated with 0.1, 1, 10 µg/ml OA in presence of 40 µM C75 (C) or 2 µM TOFA (D) or control for 24 hours after which OA and inhibitors was washed away and proliferation was measured on day 4. Data are shown as mean + SD of triplicates and are from one representative experiments out of 3-4 performed. Summary of stimulation indexes for all experiments performed are shown for both C75 (E) and TOFA (F), dots in graphs shows stimulation indexes of representative experiments shown in C or D.

leads to an overall increase in all metabolic parameters (data not shown). OA only had a modest effect on glycolysis (phosphoenolpyruvate), early TCA cycle intermediates (cis-aconitate and citrate) and metabolites involved in pyrimidine synthesis (n-carbamoylaspartate, dihydroorotate and orotate) (Fig 3A and 3B). The effects induced by OA seemed to increase at 24 hours compared to 8 hours, however, these effects were independent on the concentration of OA.

Oleic acid is not used as energetic substrate

To determine whether OA would fuel the TCA cycle and pyrimidine synthesis, ^{13}C-OA was used. Although ^{13}C-OA was taken up by the cells and partly bound to L-carnitine, no trace of ^{13}C-OA could be detected in the TCA cycle or pyrimidine synthesis (Fig 4). In combination with the fact that etomoxir did not affect the effect of OA, we conclude that beta-oxidation of OA in not involved in the ability of OA to enhance T cell proliferation.

Oleic acid is incorporated in phosphatidylcholines

We additionally used ^{13}C-OA to estimate the amount of OA that was taken up and to map the fate of OA CD4$^+$ T cells. Around 15-40% of the added OA was taken up by the CD4$^+$ T cells (Fig 5A) and was mainly incorporated into phosphatidylcholines (PCs) (Fig 5B and Suppl. Fig 1). However, treatment of cells with a higher dose of OA also led to incorporation of OA into triglycerides. Blocking the formation of newly synthesized fatty acids at two different stages, with C75 (inhibitor of fatty acid synthase (FAS)) and TOFA (inhibitor of acetyl-CoA carboxylase (ACC)) revealed that both inhibited proliferation of CD4$^+$ T cells (Fig 5C and E, respectively). Enhanced proliferation with OA treatment was still observed as demonstrated by the stimulation indexes (Fig 5D and F). These findings indicate that newly synthesized fatty acids are important for αCD3/αCD28 induced proliferation, but not for the enhanced proliferation induced by OA.

Oleic acid enhances TCR signalling in CD4$^+$ T cells

As OA could also influence the signalling and thereby enhancing the proliferation of CD4$^+$ T cells, we next determined whether TCR signalling was affected. Therefore, we determined both phosphorylation of ZAP70 and calcium fluxes in the cell, both downstream of TCR signalling. Calcium flux analyses demonstrated that baseline calcium flux is unaffected by treatment with OA. However, stimulation of CD4$^+$ T cells treated with OA leads to enhanced calcium flux responses (Fig 6A), indicated

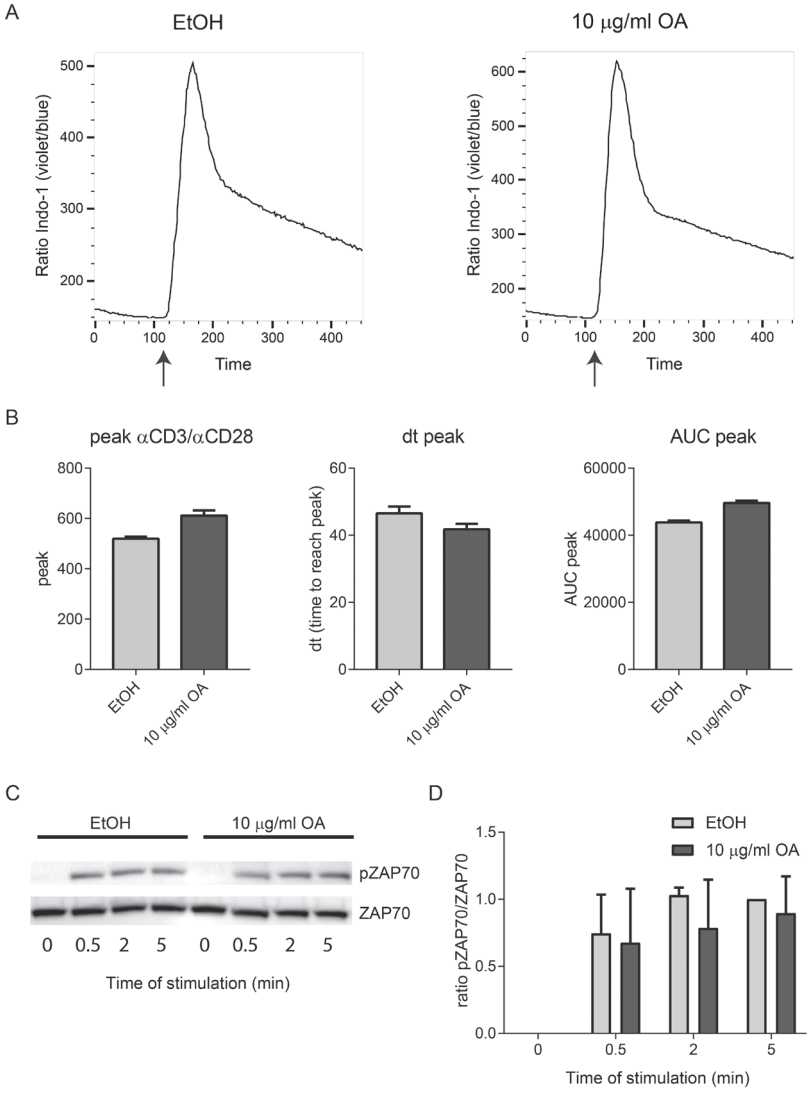

Figure 6. Oleic acid enhances TCR signalling in CD4+ T cells

Isolated CD4+ T cells were treated with 10 μg/ml OA or EtOH and after 24 hours cells were harvested, stained with indo-1 AM in the presence of 5 μg/ml αCD3 and 1 μg/ml αCD28. Basal levels of calcium flux was measured for 2 minutes after which 1 μg/ml goat-anti-mouse antibody was added to cross-link αCD3 and αCD28 (indicated with arrow) and calcium flux was measured for 5 minutes. Representative calcium flux plot is shown (A) and summary of 5 samples is shown to indicate highest point of peak in response to αCD3/αCD28, time to reach the peak and area under the curve of the peak (B). One representative experiments is shown out of 2 performed. Isolated CD4+ T cells were treated with 10 μg/ml OA or EtOH and after 24 hours cells were harvested, and incubated with 25 μg/ml αCD3 and 5 μg/ml αCD28 for 15 min at 37°C. Cross-linking was achieved by incubating cells with 5 μg/ml goat-anti-mouse antibody at 37°C for 30 sec, 2 min or 5 min. Stimulation was stopped by ice-cold PBS. Lysates were made and expression of pZAP70 (Y319) and ZAP70 was determined by western blot analysis. One representative experiment (A) and a summary of both experiments performed is shown (B).

by the calcium flux peak, time to reach this peak and the area under the curve of the calcium flux peak (Fig 6B). Stimulation of $CD4^+$ T cells induced phosphorylation of ZAP70, however, this was not affected by the treatment with OA (Fig 6C and D). These observations may suggest that although ZAP70 is unaffected, OA influences TCR triggering through enhanced calcium fluxes.

Discussion

In this study, we investigated the underlying mechanisms for enhanced proliferation of $CD4^+$ T cells in the presence of free fatty acids. Our study indicates that rather than used as a source of energy, OA is used as building block as it is incorporated into phosphatidylcholines. Moreover, it appears to influence TCR signalling indicated by enhanced calcium fluxes.

TCR triggering of $CD4^+$ T cells resulted in enhanced glycolysis and OXPHOS, as also previously described both in mice [1, 2] and humans [20]. However, treatment with OA did not influence either pathway, suggesting that OA is not used as energy source. This is in contrast to previous studies showing that affecting FAO, fatty acid uptake or biosynthesis does influence the metabolic state of T cells. Inhibition of FAO reduces OCR in memory $CD8^+$ T cells, while OCR of $CD8^+$ effector T cells was unaffected [21]. Furthermore, inhibition of fatty acid uptake or synthesis in naïve stimulated $CD4^+$ T cells decreased both OCR and ECAR, while OCR of Th2 cells was unaffected by these treatments [15]. This suggests that the effect of fatty acids on the metabolism of $CD4^+$ T cells is dependent on the differentiation state of the cells.

In line with recent findings [15], we showed that OA is incorporated in PCs, which are main components of the cell membrane. It remains unknown, whether these OA-containing PCs are used to generate new membranes, replacing the PCs already present in the membranes or in which membranes these OA-containing PCs are incorporated. However, as we have observed that blocking the synthesis of new fatty acids on two different stages (TOFA and C75) did not influence the stimulation index of OA this suggests that OA is incorporated in existing membranes.

Incorporation of OA-containing PCs in the membrane could influence membrane fluidity and subsequently could influence TCR signalling [11, 22]. We have also

observed that calcium flux is enhanced with treatment with OA, suggesting that TCR signalling of the cells was influenced, however, phosphorylation of ZAP70 was not influenced. Together with lymphocyte-specific protein tyrosine kinase (Lck), ZAP70 is one of the first steps of TCR signalling, leading to phosphorylation of linker for the activation of T cells (LAT), which recruit many other signalling molecules, such as phospholipase C gamma1 (PLCγ1) [23]. PLCγ1 signals induces intracellular calcium release, which serves as the primary trigger for the opening of calcium channels in the plasma membrane [24]. Although phosphorylation of ZAP70 was not influenced by OA treatment, other TCR signalling molecules such as LAT or PLCγ1 could still be affected. Therefore, future studies should investigate whether other molecules downstream of TCR signalling, such as LAT and PLCγ1, are affected by OA treatment and therefore contribute to higher calcium fluxes.

Furthermore, our metabolomics study showed that OA-treated CD4+ T cells have enhanced pyrimidine synthesis, which is supported by the observed elevated levels of incorporated ^{3}H-Thymidine. Pyrimidines not only support DNA synthesis, but pyrimidines also control the progression through the S phase of the cell cycle [25], suggesting that treatment with OA could influence this, resulting in enhanced proliferation of CD4+ T cells.

The increased proliferation of T cells in the presence of oleic acid could be relevant for inflammatory conditions associated with obesity, which is usually accompanied by increased free fatty acid levels. One example is the joint of osteoarthritis patients with a high BMI, in which there is a higher release of free fatty acids from the infrapatellar fat pad [26], likely resulting in high levels of free fatty acids in the joint. The enhanced proliferation of the joint T cells in a high free fatty acid environment could underlie the observed correlation between the frequency of CD4+ T cells in synovium and BMI [27].

On the other hand, increased free fatty acid levels in the microenvironment could also lead to accumulation of intracellular lipid droplets. These have been recently associated with increased tissue invasiveness of T cells in rheumatoid arthritis, resulting in increased inflammation [28]. Although a full understanding of the effects of fatty acid uptake on T cell function is still lacking, these data indicate that interfering with free fatty acid synthesis / uptake could constitute an effective therapeutic approach in obesity-related diseases.

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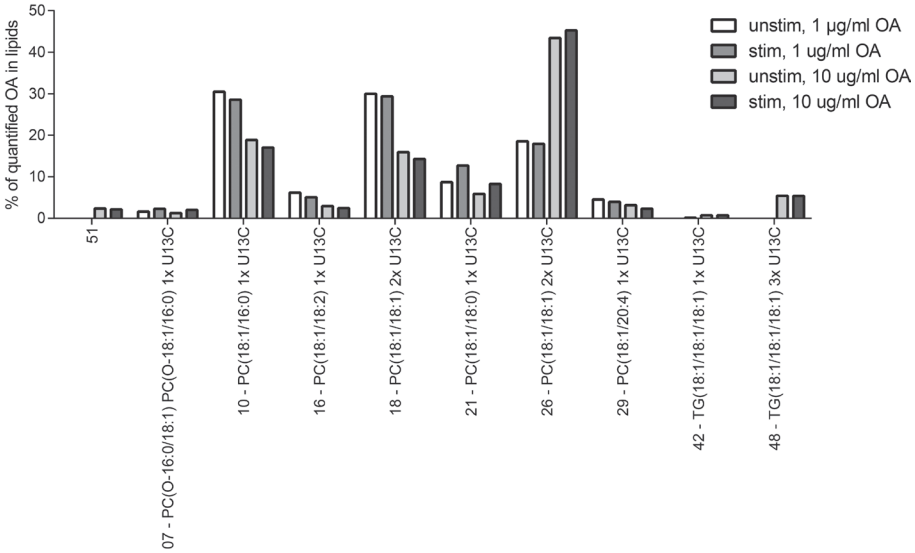
Supporting information



Supplementary figure 1. Oleic acid is incorporated in different phosphatidylcholines

Isolated $CD4^+$ T cells were treated with 1 or 10 µg/ml 13C-OA in presence or absence of pb. αCD3 and sol. αCD28 for 24 hours and LC-FT/MS was used to determine in which higher order lipids 13C-OA was incorporated. After we selected the top ten of lipids which contained the highest intensity of 13C-OA, we determined the relative amount of OA incorporated in these ten lipids. The sum of 13C-OA in these ten quantified lipids was used as total amount (100%).