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Reprogramación metabólica de macrófagos inducida por dislipidemia en retinopatía diabética

TESIS

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MAESTRA EN CIENCIAS

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ABSTRACT

DR is a major neurodegenerative, microvascular, and inflammatory complication of Diabetes Mellitus (DM) and the leading cause of visual impairment in working age population. Inflammation contributes to the development and progression of Diabetic Retinopathy (DR). Combination therapies of fenofibrate (PPARα inhibitor) and simvastatin (and HMG CoA reductase inhibitor) in addition to glucose control have demonstrated encouraging results at reducing overall complications of T2DM. Despite efforts to trying link dyslipidemia and development and progression of DR, results have not been conclusive so far. Our data show that lipids found in the plasma of T2DM patients induce an upregulation of lipid storage and metabolism-related genes in monocytes (Mo). Such activated Mo are found in the retina of patient with DR in the vicinity of active leakage. We showed that lipid dependent Mo activation is associated with a complex metabolic reprograming, associated with the secretion of inflammatory cytokines. Finally, our results suggest that compounds inhibiting PPAR pathway as well as drug targeting key enzyme in lipid metabolism blunt Mo activation in a lipid-rich environment. Our data support a model in which impairment of the Blood-Retina-Barrier (BRB) leads to extravasation of plasma content (lipids and proteins) and leukocytes (including Mo); this lipidic environment leads Mo to differentiate into pro-inflammatory macrophages (Mϕ) trough metabolic reprogramming and to further participate in neuroinflammation of the DR retina.

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A quien corresponda:

Por medio de la presente autorizo la tesis: "Reprogramación metabólica de macrófagos inducida por dislipidemia en retinopatía diabética".

Elaborada por la alumna Frida Paulina Muñiz Ruvalcaba, con No. de cuenta 310589077, adscrita al programa de maestría internacional en ciencias de la Visión Sorbonne-UNAM. Dicha tesis se realizó en el marco colaborativo entre el laboratorio de Biología Molecular de la escuela de medicina de la universidad Panamericana a mi cargo y el laboratorio a cargo del Dr Xavier Guilloneau del Institut de la vision París, Fr.

Me despido envándoles un saludo cordial

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PREFACE

This work was realized with results obtained in the months of October and till the beginning of the confinement (February and mid-March). Some results were previously obtained by the host team, result analysis and reanalysis were performed at stay-at-home work. Result discussions were accomplished by videoconference meetings.

Results from the month of October were mostly qPCRs and the experiment settings and conditions were already stablished. For the experiments preformed in the months of February and March, new sets of conditions were stablished, and most plates were stored in order to further analyze them. The quickest way of obtaining results were through IL-6 ELISA tests to further analyze the potential drugs that reduced IL-6 secretion in the gene regulation, this was not possible because of the situation this experiments could not be performed neither repetition of the ELISA test in order to obtain statistical significance.

Acknowledgments:

To my family for always supporting me in my crazy decisions, being a moral and emotional support, as well somebody I can always relies on.

To my boyfriend, for always given me advice and support, for always being form me and advise me in personal and academic situation.

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To my friends for being someone who I can rely on even though distance and time differences made it difficult. For making difficult times more bearable and always make me smile.

CONTRIBUTION

This project was realized at the Institut de la Vision in Paris, France in the team of Florian Sennlaub. The group leader and project director was Xavier Guillonneau. The experimental and review of the project was done with the help and trainning of Doctoral student Guillaume Blot. In the following lines I will describe the contributions to the development of this report.

Xavier GUILLONEAU: Experiment design. Training for the experiments. Report review and correction

Guillaume BLOT: Experiment design. Training for the methodological experiments. Experiments related to culture of Mo in the presence of hi-plasma, including statistical analysis. Confocal Image acquisition of Immunohistochemistry samples. Experiments of Mo in the presence of PA at different concentrations. Mo isolation and culture in the presence of PPAR agonists/antagonists as well as RNA extraction of these experiments. Human eye dissection Report review and correction

Frida Paulina MUÑIZ RUVALCABA: Immunohistochemistry of DR retina tissue samples and image analysis. Monocyte isolation for ELISA and Seahorse experiments. RNA isolation qPCR, data acquisition and analysis for hi-plasma T2DM experiments. RNA isolation qPCR, data acquisition and analysis for PPAR agonist/antagonist experiments. IL-6 ELISA test and statistical analysis. Report writing.

ABREVIATIONS

ACCORD: Action to Control Cardiovascular Risk in Diabetes

AGE: advanced glycation end-products

BRB: Blood-Retina-Barrier

BSA: Bovine serum albumin

DM: Diabetes mellitus

DME: Diabetic macular edema

DR: Diabetic retinopathy

ECAR: Extracellular acidification rate

FA: Fatty acids

FFA: Free fatty acids

FIELD: Fenofibrate Intervention and Event Lowering in Diabetes

LDL: Low density lipoproteins

Mϕ: Macrophages

Mo: Monocytes

MP: Mononuclear phagocytes

NG: Normal glucose

NPDR: Non proliferative diabetic retinopathy

OCR: Oxygen consumption rate

OXPHOS: Oxidative phosphorylation

PA: Palmitate

PA-Br: Bromopalmitate

PA-CH3: Methylpalmitate

PDR: Proliferative diabetic retinopathy

PFA: Paraformaldehyde

PPAR: Peroxisome proliferator receptor

RPE: Retinal pigmented epithelium

T2DM: Type 2 diabetes mellitus

BACKGROUND

DM is a metabolic disease and a major health problem, affecting approximately 422 million people and projected to rise to 629 million by 2045. DR is a microvascular complication of DM and one of the leading causes of blindness in working-age adults[1].

The retina is a multilayered tissue from embryonic neuronal origin; There are five types of neurons in the retina (photoreceptors, bipolar cells, ganglion cells, horizontal cells and amacrine cells), structurally organized in order to form the 10 layers of the retina[2]. The neuro-retina processes information and send it through the optic nerve to the visual cortex, other cells such as the RPE cells, endothelial cells, pericytes, Müller glia, astrocytes and microglia maintain retinal functions, such as support, nutrient transport and surveillance[3] .The BRB is divided in two: i) the inner BRB formed by the tight junctions of the endothelial cells from the blood vessels and ii) the outer BRB, constituted by the tight junctions of the RPE cells. The association between neurons, glia and pericytes with the vascular endothelial cells and extracellular matrix is called the neurovascular unit (NVU). These interactions between cells work coordinately as a whole, providing a regulated environment that allows proper neuronal function[4,5]. The properties of the tight junctions make the BRB a restrictive barrier only allowing the access to ions, proteins, and water[6]. In the outer part of the retina, in the basal lamina there is the Bruch's Membrane which supports the RPE, followed by the choroid which is a vascular tissue that provides nutrients and oxygen to the outer retina and RPE[7]. The inner retina receives the vast majority of blood supply by the ophthalmic artery which runs superior to the optic nerve where one of its major branches is the central retinal artery[8]. Lastly the BRB possesses an immunoregulatory environment proportionated by pericytes, which prevents extravasation of circulating leukocytes into the retina[9].

DR has been long recognized as a microvascular, inflammatory, and neurodegenerative disease[10]. DR progresses from NPDR (characterized by capillary occlusion, vessel regression and inflammation) to PDR, characterized by the growth of abnormal new retinal blood vessels[11]. At any DR stage there can be the presence of exudates identified as white-yellow speckles constituted of lipid depositions due to plasma leakage from damaged capillaries[12]; and DME characterized by fluid leakage in the macula (including leukocytes, plasma proteins, glucose and lipids) due to disruption of the BRB[13].

Hyperglycemic conditions in DR contributes to the increase in the polyol pathway flux and hexosamine biosynthesis, hyperosmolarity, advanced glycation end-products (AGE) formation,

signaling pathway dysregulation such as activation of protein kinase C (PKC) pathway, all together lead to oxidative damage to the inner walls of the retinal capillaries (causing their occlusion) and upregulation of proinflammatory cytokines[14,15,16]

Tight control of blood glycemia remains the first line treatment of T2DM and studies where intensive glycemic control treatment was performed at early diagnosed T2DM patients have showed that the overall risk for any complication was significantly reduced[17,18]. However an increased cardiovascular risk was observed in studies where patients were assigned to an intensive glycemic control treatment[19] and a rapid normalization of glycemia was shown to worsen the DR[20]. Finally, analysis of the Diabetes Control and Complications Trial data reported that glycemic exposure only explains approximately 11% risk for DR development and that the remaining 89% may be due to other factors[21]. All together, these clinical studies pointed out the necessity to reduce T2DM risk factors other than hyperglycemia. The FIELD Study showed that combination therapy with fenofibrate (a lipid lowering drug), reduced the need for laser photocoagulation[22], while the ACCORD Eye Study showed that fenofibrate in combination with simvastatin (a cholesterol lowering drug) reduced the progression of DR at early stages[23]. In addition to this, it has been reported a correlation between hard exudates and serum lipid levels[24], as well as perivascular deposition of lipid-laden macrophages in the retina of patients with DR[25] suggesting that lipid may trigger retinal neuroinflammation leading to vascular remodeling. To date, little is known about the mechanisms linking dyslipidemia and inflammation to DR progression, but the encouraging result from the ACCORD and FIELD studies suggest that a better control of lipids during DR or the use of pharmaco-therapy to limit the effects of lipids on retinal cells may provide an alternative therapy rather than to blood sugar and blood pressure control alone, and help a reducing the need of laser treatment[26]

Additionally to genetic predisposition, the main risk factors for the development of T2DM are sedentary life, elevated body mass index and obesity, which are indicative of an increased intra-abdominal visceral fat, leading to disruption of insulin metabolism[27]. Several groups have studied the correlation between triglycerides and cholesterol levels to DR, the results have not yet been conclusive, however the use of statins have been effective at preventing the development of NPDR[28]. LDL and modified lipoproteins have been shown to have an indirect correlation between DR progression and DME, as for the correlation between plasma lipid profiles have shown significance but not enough strength as to be consider a risk factor for DR progression and development, and DME occurrence[29,30]. All together these study demonstrate

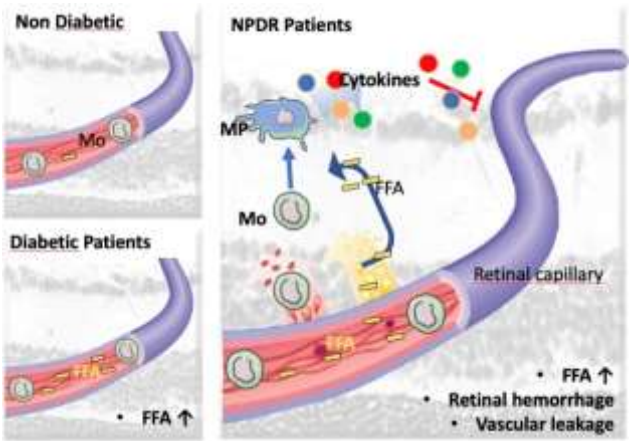
that despite intensive effort to link DR progression to a particular class of lipids, results have not been conclusive so far[31,32].

The role of FFA and impairment in lipid metabolism in retinal diseases have just been emerging. FFA have been demonstrated to be elevated in plasma samples of patients with T2DM, and to be the causal agent for developing atherosclerosis, insulin sensitivity and DR[33,34]. Though the role of different FA in health and pathology have just started to be understood, this field requires study[31].

DR is an inflammatory disease, findings suggest that inflammation is the first step leading to further complications, as it has been reported elevated levels of proinflammatory biomarker such as vascular adhesion molecules, pro- and anti- inflammatory molecules, as well as angiogenic and anti-angiogenic chemokines, and growth factors, both systemic and peripherally[35,36]. Hyperglycemia contributes to an increase in the expression of ICAM-1, further leading to the recruitment of leucocytes around the vascular walls of retinal capillaries followed by the release of proinflammatory cytokines[37,38]. There is evidence that inflammation and peripheral monocyte (Mo) infiltration plays a critical role in DR progression[39]. It has been reported elevated concentrations of CCL2 (also known as MCP-1) in the vitreous of patients with diabetic retinopathy[40]. Consistent to this data, CCL2 polymorphisms has been proposed as a potential risk factor for DR development and progression[41,42]. The ability of FFA to polarize Mo into pro- and anti-inflammatory Mϕ has been demonstrated, though the underlying mechanisms are still not well understood[43,44]. PA the most studied FFA has been proved to polarize Mo to a pro-inflammatory profile, at first it was thought to be though TLR4 receptor, but recently it has been proved this is not the case, giving rise to more questions on the mechanisms that leads to dyslipidemia Mo activation[45].

HYPOTHESIS

The contribution of dyslipidemia to DR pathogenesis is now widely accepted and supported[22,46]. However, there are still discrepancies between the association of the different lipid class found in blood and DR progression reported in the literature so far. It suggests that the link between dyslipidemia and DR is complex and



remains poorly understood[26]. An unexpected link might be that in the compromised DR retina, BRB disruption and immune privilege loss contribute to an unconventional situation where monocytes differentiate in a lipid rich environment. Indeed, retina from non-diabetic individuals (Figure A) and T2DM patients which have elevated circulating lipid level (Figure B) are protected from plasmatic lipid variation by the intact BRB while BRB impairment in DR patients allow simultaneous extravasation of plasmatic lipids and monocyte in the retina (Figure C). We hypothesize that the abnormal dyslipidemic retinal environment in DR modify the metabolism of infiltrating monocytes and force their differentiation into pro-inflammatory Mϕ.

OBJECTIVE

To determine if lipids found in the plasma of T2DM/NPDR patients alter monocyte differentiation, we will: 1) Determine if Mϕ found in the retina of DR patient expresses markers that reflect the use of lipid. 2) Assess the differentiation profile (pro-inflammatory cytokine secretion, glycolysis and lipolysis) of Mo differentiated in the presence of plasma from T2DM patient (including NPDR and DR patients) or plasma from CTL donors and compare it to that of the differentiation in the presence of the FFA palmitate (PA) to evaluate the role of lipid in Mo polarization. 3) Analyze the metabolic changes in Monocytes treated with FFA by Seahorse analysis, and 4) Evaluate the ability of different drugs targeting glucose and lipid metabolism as well as the PPAR family to normalize Mo differentiation in a lipid rich environment.

All together could lead to the development of new therapy avenues in order to prevent DR development and progression for DR treatment.

METHODS

Immunohistochemistry

Donor eyes with known history of DR and controls were collected through the Minnesota Lions Eye bank (1 control retina from 1 donor; 4 retinas with DR from 2 donors). Posterior segment was fixed 4 h in 4% Paraformaldehyde and dissected. Retina samples of 5 mm diameter were obtained with a biopsy punch, fixed in PFA 4% and stored ant –80 C until use. Labeling was performed using the indirect immunofluorescence method for free floating tissue. IBA-1 (Rabbit-Anti-Human), PLIN2 (Mouse-Anti-Human), Albumin (Mouse-Anti-Human), Col-IV (Goat-Anti-Human) immunohistochemical analysis were performed and revealed using appropriated fluorescent coupled secondary antibodies. Tissue samples were flat-mounted.

Image acquisition was done with an Olympus confocal microscope and image analysis was performed with Imaris Viewer Software (Oxford Instruments, UK).

Plasma samples from T2DM patients

Plasma samples from patients with T2DM, NPDR, PDR and healthy donors were obtained from a stablished cohort of the Institute of Ophthalmology Foundation Conde de Valenciana (Mexico City, Mexico) with approval from local committees.

Monocyte isolation

Human naïve Mo were isolated from peripheral blood of healthy volunteer donors by a density gradient performed with Lymphoprep (Stemcell Technologies, Canada). Once peripheral blood mononuclear cells were isolated, negative selection of Mo was performed with EasySep Human Monocyte Enrichment Kit (Stemcell Technologies, Canada).

Mos Treatment

Isolated human Mo were cultured in NG DMEM medium (DMEM 5 mM glucose, 20 mM mannitol, BSA 0.88 w/v, etOH 0.5 v/v) or NG/PA (DMEM 5 mM glucose, 20 mM mannitol, BSA 0.88 w/v, PA 250 µM, etOH 0,5 v/v) 250 µM with or without Insulin 100 nM, Metformin 100 µM, Insulin 100 nM + Metformin 100 µM, (R)-(+)-Etomoxir sodium salt 100 µM, Tricasin C 3 µM, GW6471 2 µM, PA-CH3 250 µM, PA-Br 250 µM, GW9662 10 µM. Monocytes were cultured in a 37°C, 5% CO_2 incubator for 18 h, conditioned media were collected and stored at –80 C and cell lysed with LB1 lysis buffer (Macherey-Nagel GmbH & Co. KG, Germany) for further use in RT-qPCR.

RNA isolation, reverse transcription (RT) and quantitative real-time PCR (qPCR)

RNA isolation was performed with NucleoSpin® RNA XS RNA isolation kit (Macherey-Nagel GmbH & Co. KG, Germany) according to the manufacturer's instructions, quantified with a spectrophotometer (NanoDrop ND-2000; Thermo Scientific, USA). cDNA was synthesized by reverse transcription from total RNA using QuantiTect® Reverse Transcription kit (QIAGEN Sample and Assay Technologies, Germany). qPCR was performed on the first-strand cDNA synthesis reaction products, using Specific primers in Table 1 (Anexes) and Sybr Green PCR Master Mix (Life Technologies) in StepOne Plus real-time PCR system (Applied Biosystems) with the following profile: 10 min at 95°C, followed by a total of 40 two-temperature cycles (15 sec at 95°C and 1 min at 60°C). Specificity of all primers was assessed by examination of

the dissociation curve. Results were expressed as fold-change relative to endogenous control calculated using ΔΔCt method.

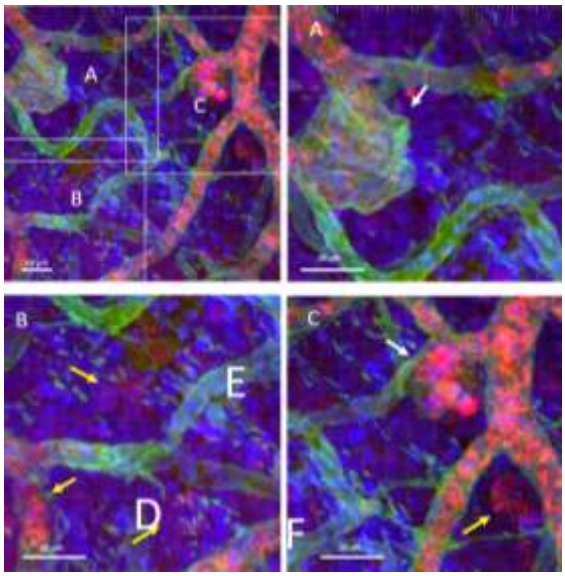


Fig. 1. *Confocal microscopy image of retina tissue from a patient with DR; nuclei (Hoechst), Col-IV (Green), Albumin (Red). Enlargement to interest zones are represented with letters (**A, B, C**) Pointing micro-aneurisms (White arrows), Hemorrhage (yellow arrows). Scale bar 20 µm.*

Metabolism assessment

Real time metabolic assessment was performed on human Mo pretreated with NG/DMEM medium, PA 250 µM and Methylpalmitate (PA-CH3) 250 µM on a Seahorse XFp Analyzer (Agilent Technologies, USA) according to the manufacturer's protocol. Data analysis was performed with Seahorse Wave Desktop Software (Agilent Technologies, USA).

Enzyme-linked Immunosorbent Assay (ELISA)

Human IL-6 cytokine concentrations in monocyte conditioned media from different culture conditions were quantified with Quantikine ELISA assay (R&D Systems a bio-techne brand, USA) following the manufacturer's protocol, the colorimetric reaction was determined by spectrophotometry (Spark, TECAN Trading AG, Switzerland).

RESULTS

Activated MP can be found near hemorrhages in retina of DR patients

Immunohistochemistry of free floating, flat mounted retinas from patients with DR were performed to evaluate the presence and relationship between microaneurysm, plasma leakage and inflammatory cells. Confocal imaging was first performed to analyze tissue vasculature stained with collagen IV and particular attention was paid to the presence of microaneurysms (focal dilatation of retinal capillaries) (**Fig. 1A**, white arrows). To investigate potential hemorrhages, we performed staining of albumin, the most abundant plasma protein. As seen in **Fig. 1B, C** (yellow arrows) albumin can be observed in the retinal capillaries, but also outside the vessel as demonstrate by a cloudy speckled staining. In **Fig. 2** we can appreciate the

presence of Iba-1 (Green)/PLIN2 (Red) double positive cells (microglia or Mϕ, white arrowheads) near microaneurysms, as PLIN2 is a protein involved in lipid storage, it suggests that activated microglia/Mϕ may participate in a process of lipid internalization and storage. We next analyzed the presence of MP in the vicinity of vascular hemorrhages by immunostaining of Albumin (Red), Col-IV (Grey) and Iba-1 (**Fig. 3**) Iba-1 positive cells are located near vessels, and extra-vascular albumin deposit, although the presence of microaneurysms were not detected for this particular tissue region

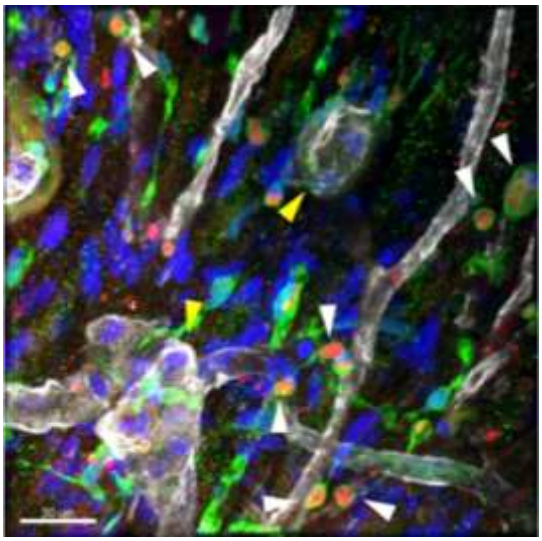


Fig. 2. *Confocal microscopy image of retina tissue from a patient with DR; nuclei (Hoechst), Col-IV (Grey), Iba-1 (Green), PLIN2 (Red). Micro-aneurisms (yellow arrowheads) and Iba-1/PLIN2 double positive cells (white arrowheads). Scale bar 50 µm.*

Plasma from T2DM causes dysregulation of pro-inflammatory cytokines and lipid metabolism related genes in Mo

In DR, BRB disruption leads to plasma (including lipid) and leucocytes (among them monocytes) extravasation in retinal tissue. We hypothesize that Mo-derived Mϕ differentiation in a lipid rich environment drive them to a pro-inflammatory activation which could lead to the progression of the disease. We first investigate the polarization profile of Mo when cultured in presence of healthy (CTL), or T2DM (T2DM with no sign of DR, NPDR or PDR) donor heat-inactivated (hi) plasma, with particular emphasis on genes known to be overexpressed in DR retina (*VEGF, CCL2, IL8, ANGPTL4*) and genes implicated in lipid storage and metabolism (*PLIN2, PDK4, ACADVL*).

mRNA expression of proinflammatory genes such as *IL-8* and *CCL2* normalized with *RPS26*

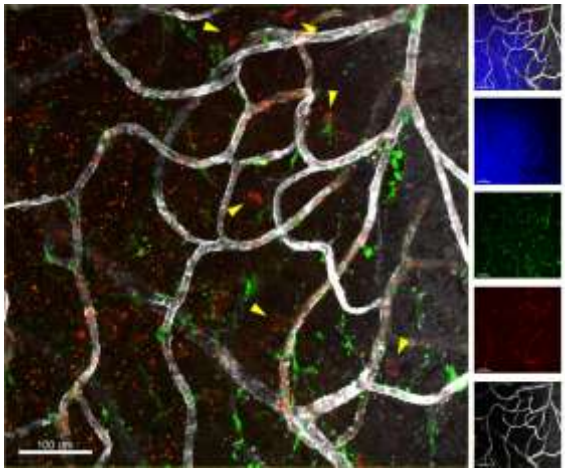


Fig. 3. *Confocal microscopy image of retina tissue from a patient with DR; nuclei (Hoechst), Col-IV (Grey), Iba-1 (Green), Albumin (Red). Hemorrhages (yellow arrowheads). Lateral images single channel image acquisition and merge image. Scale bar 50 µm.*

mRNA in plasma samples of T2DM showed no statistical difference in comparison to control group ($p<0.05$) (**Fig. 4A & B**). Genes involved in lipid metabolism such as *PLIN2* (storage), *ANGPTL4* (regulation of glucose homeostasis, lipid metabolism and insulin sensitivity), *PPARA* (adipogenesis and inflammation), *PDK4* (regulation of glucose and fatty acid metabolism and homeostasis), *ACADVL* (catalyzes the first step on mitochondrial fatty acid beta-oxidation pathway) were upregulated in comparison to control group ($p<0.05$) (**Fig. 4C-G**). These results are consistent with Illumina RNAseq of healthy Mo treated with FFA previously obtained by the host team, showing increase in gene expression of *VEGF*, proinflammatory and lipid storage and metabolism related genes (unpublished data). No differences between subgroups of T2DM (T2DM without DR, NPDR and PDR) were observed

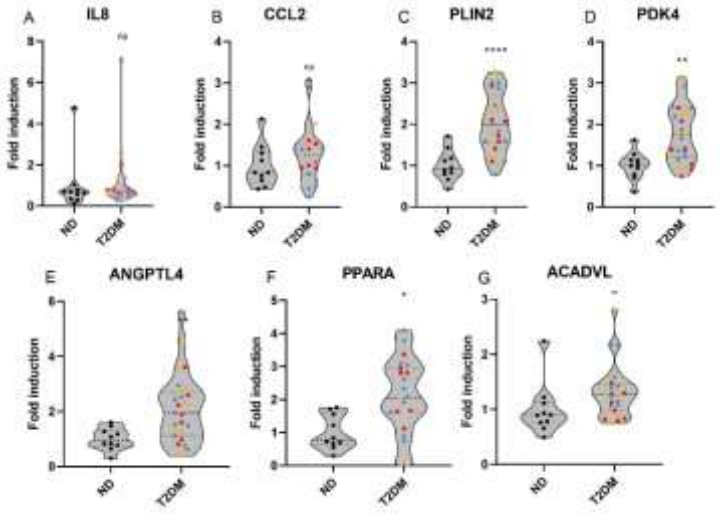


Fig. 4. *Cytokine and Lipid metabolism gene expression in of Mo seeded in the presence of hi-plasma samples from T2DM patients.*

RT-PCR of relative mRNA expression normalized with RPS26 mRNA of Mo cultured in the presence of hi-plasma samples from T2DM patients. All groups except for IL-8 and CCL2 were significantly different by two-way Kruskal-Wallis ANOVA p<o.o5. ND (black dots), T2DM (blue dots), NPDR (yellow dots), PDR (red dots).

Palmitate increases the expression of proinflammatory and lipid metabolism related genes in a dose dependent manner in primary human Mo culture

One of the most abundant lipidic components in plasma of T2DM are FFA, its most representative one is Palmitate (PA). It has been previously reported the capacity of PA in Mo activation and subsequent release of pro-inflammatory cytokines[47]. In order to evaluate the concentration at which Mo would be activated an produce a similar profile to that found with

hi-plasma of the DR cohort we challenged primary human Mo with concentrations of PA ranging from 50 µM to 500 µM. qPCR analysis showed that PA induced the upregulation of pro-angiogenic(*VEGF*; **Fig. 5D**), anti-angiogenic (*ANGPTL4*; **Fig. 5E**), pro-inflammatory (*CCL2, IL-8, IL-6*; **Fig. 5A-C**) and, lipid storage and metabolism (*PDK4, ACADVL*; **Fig. 5F & G**) related genes in a dose-dependent manner. All genes were induced at 250 µM, this concentration was thus selected for further experiments

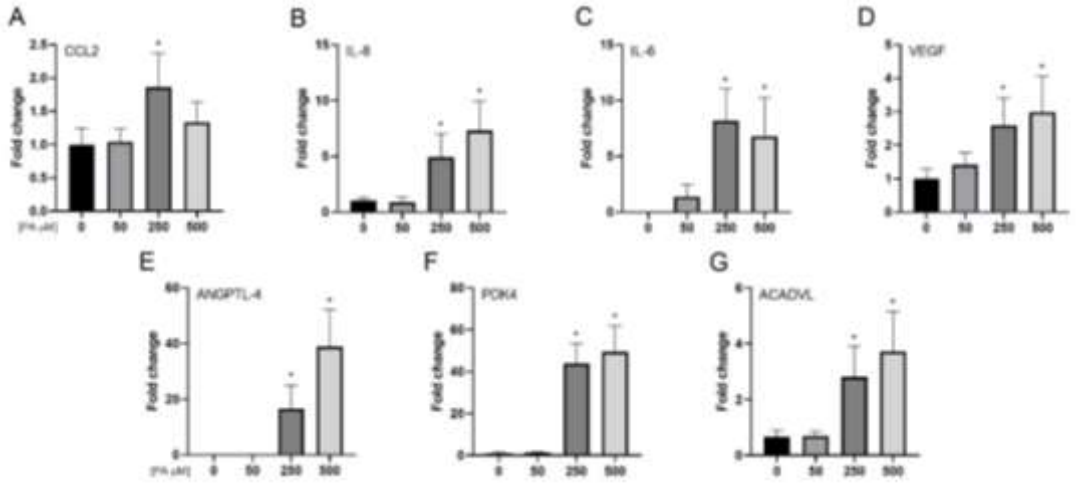


Fig. 5. *RT-qPCR of relative mRNA of pro-inflammatory (**A, B, C**), angiogenic (**D**), anti-angiogenic (**E**) and lipid metabolism (**F & G**) related genes expression normalized with RPS26 mRNA of primary human monocytes seeded in the presence of increasing concentrations of PA, ranging from 50 µM to 500 µM. Statistical difference Turkey's ANOVA ($p<0.05$) in comparison to 0 µM and 50 µM PA concentration.*

Metabolism assessment of primary human Mo

The overexpression of lipid metabolism related genes observed both with the T2DM cohort and primary human Mo in the presence of PA, suggests a shift from glucose to lipid metabolism. We aimed at determining whether PA is used as fuel source for TCA/OXPHOS-linked ATP production in primary human Mo. To this end, on a Seahorse analyzer, we performed a real-time measurement of oxygen consumption rate (OCR) indicating use of O_2, and a real-time of extracellular acidification rate (ECAR) determination, which indicates extracellular proton secretion.

Prior to Seahorse analysis, Mo were treated for 18 hours with either BSA, PA or methylpalmitate (CH_3-PA), a non-metabolizable analogue of PA, allowing intracellular uptake of lipid source. Basal OCR levels were found reduced when Mo were pretreated with PA

compared to the control groups (BSA or CH_3-PA) (**Fig. 6A**). After successive injection of Oligomycin (an ATP synthase inhibitor) and Rotenone/Antimycin A (inhibitors of mitochondrial complex I and complex III, respectively) the OCR level reached similar levels in all conditions, indicating an equal non mitochondrial oxygen consumption (**Fig. 6A**). Potential mitochondrial O_2 consumption not linked to ATP production (mitochondrial H^+ leak, reactive oxygen species generation) is determined by the difference of OCR level after oligomycin-only injection and after Oligomycin + Rotenone/Antimycin injection. Our results indicate very little mitochondrial O_2 consumption not linked to ATP production in all groups (**Fig. 6A**).

In contrast, basal ECAR levels were found elevated in the PA pretreated Mo group compared to the control groups (BSA or CH_3-PA) (**Fig. 6B**) after addition of oligomycin when TCA/OXPHOS-linked ATP production is inhibited. There is a compensatory increase of aerobic glycolysis for ATP production here, observed by an increase of ECAR. Interestingly, after oligomycin addition ECAR rise is more important in control group than in PA pretreated Mo, indicating a lower glycolytic reserve in this condition (**Fig. 6B**). The Seahorse test we performed is not designed for further ECAR data interpretation after injection of Rotenone/Antimycin A.

Since PA-pretreated Mo use less TCA/OXPHOS, the higher ECAR basal level could originate from a more important aerobic glycolysis (with lactate as end product). All together these results indicate a clear metabolic modification in Mo treated with PA. PA induces a reduction in the consumption of oxygen and a higher acidification.

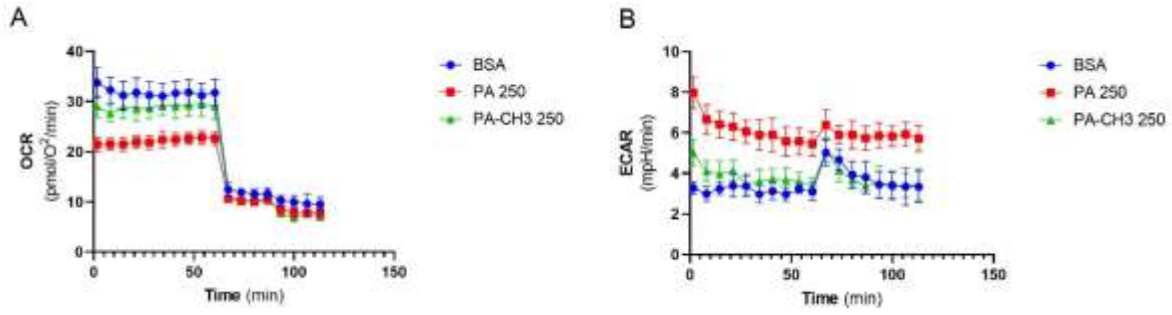


Fig. 6. *Metabolism assessment of human primary monocytes by Seahorse Analysis.*

A) *Oxygen consumption rate (OCR) of human primary monocyte culture plated with BSA (blue), PA 250 µM (red) and PA-CH3 250 µM (green). Arrows indicate the administration of Oligomycin 1.5 µM (at 60 min) and Rotenone µM /Antimycin 0.5 µM (at 80 min).*

B) *Extracellular acidification rate (ECAR) of human primary monocyte culture plated with BSA (blue), PA 250 µM (red) and PA-CH3 250 µM (green). Arrows indicate the administration of Oligomycin 1.5 µM (at 60 min) and Rotenone µM /Antimycin 0.5 µM (at 80 min).*

Evaluation of different compounds to blunt inflammatory response

Lipid lowering therapies have been proposed to reduce the risk of developing DR, or prevent its progression, but to date only fenofibrate (a PPARα activator) and simvastatin (an 3-hydroxi-4-metilglutaril CoA reductase A inhibitor) have showed encouraging results to treat DR. PPARs have an important role in inflammation, lipid storage and metabolism. In line with this, previous results of RNAseq analysis (Data not shown) showed an increased expression of PPARγ making it an area of study. We hypothesized that PPARs have a role in either lipid-induced or lipid-independent activation of Mo into pro-inflammatory Mϕ.

First, we decided to setup a test to easily quantify Mo activation in the presence of potential therapeutic agents. IL-6 production, a prototypic pro-inflammatory cytokine, was chosen as a readout of activation and its concentration was determined in the supernatant of Mo seeded in the presence of PA 250µM, PA-CH3 250 µM or PA-Br 250 µM by an ELISA assay. Our results showed that PA induced a 6-fold increased secretion of IL-6 by Mo while PA-CH3 and PA-Br groups resulted in a limited 1.5 and 2 fold increase respectively (**Fig. 7A**) (due to the lack of repetitions significance cannot be determined).

Having established a test of Mo activation, we next determine the potential detrimental role of the glycolytic metabolism in the activation of Mo in a lipid rich environment. Primary human Mo were challenged for 18h with PA 250 µM in the presence of Insulin and/or Metformin which are molecules involved in the glycolytic metabolism. To determine the potential of these compounds to blunt the secretion of pro-inflammatory cytokines, we quantified IL-6 concentration in supernatants and expressed it as a fold change to PA **(Fig. 7A)**. We showed that insulin or insulin sensitizer metformin did not modify the secretion of IL-6 in PA-stimulated Mo. However the combination of Insulin + Metformin increased the secretion of IL-6 up to 2 fold. (**Fig. 7B**) (due to the lack of repetitions significance could not be determined).

Similarly, to determine the role of lipid metabolism in Mo activation, we challenged primary human Mo with PA 250 µM in the presence of lipid metabolism inhibiting drugs. Etomoxir an inhibitor of carnitine palmitoyl transferase 1 and Triacsin C an Inhibitor of long chain acyl CoA synthase were chosen and added to PA-stimulated Mo. Our preliminary results showed that

Etomoxir and Triacsin C reduced the relative expression of IL-6 that of a 67% and 43 % respectively (**Fig. 7C**) (due to the lack of repetitions significance could not be determined).

Finally, PPARs have a role in adipogenesis and inflammation and PPARα and PPARγ are both expressed in Mo (with PPARγ having a higher expression than PPARα)[48]. We hypothesized that PPAR modulators could blunt the inflammatory response to PA. We thus challenged primary human Mo with PA 250 µM in the presence of GW6471 (PPARα antagonist) or GW9662 (PPARγ antagonist) and quantified IL-6 secretion. Our preliminary results showed that GW6471 as well as GW9662 reduced modestly IL-6 secretion (19% and 33% respectively) (**Fig. 7D**) (due to the lack of repetitions significance could not be determined).

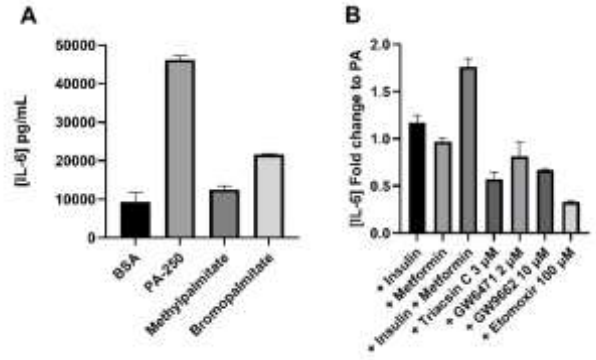


Fig. 7. *IL-6 concentration in supernatant of human primary monocytes.*

A) *IL-6 concentrations in supernatant of human primary Mo seeded in the presence of BSA, Palmitate 250 µM, PA-CH3 250 µM and PA-Br 250 µM. No statistical analysis was performed due to lack of repetitions.*

B) *IL-6 concentration fold change compared to PA IL-6 of concentrations in supernatant of human primary Mo seeded in the presence of glycolysis related compounds (Insulin and Metformin) lipid metabolism related inhibitors (Etomoxir and Triacsin C) and PPARs agonists/antagonists. No statistical analysis was performed due to lack of repetitions.*

Evaluation of different PPAR agonists/antagonists to reduce the expression of genes related to DR profile

We showed that modulators of PPARs altered IL-6 expression in PA-treated Mo. To confirm the ability of different PPARs agonists/antagonists to prevent Mo polarization into pro-inflammatory Mϕ, we further evaluated the ability of other PPARs agonist/antagonist to prevent the overexpression of genes found upregulated in DR (due to the particular situation the same modulators could not be tested in the two set of experiments). Primary human Mo were seeded

in the presence of PA at a concentration of 250 µM or 500 µM, with or without Fenofibrate (PPARα agonist), Pioglitazone (PPARγ agonist) and T007092 (PPARγ antagonist). Fenofibrate and Pioglitazone did not modify the overexpression of pro-inflammatory *IL-8* and *CCL2* nor the lipid metabolism *PPARG* and *PLIN2* related genes (**Fig. 8A-D**) ($p<0.05$). In contrast T007092 reduced the expression of *IL-8* and *IL-6* at a concentration of 12.5 µM, and of *CCL2* expression at a 25 µM concentration. Of note, the reduction of these pro-inflammatory gene expression was not dependent of the PA stimuli (**Fig**. **8E-G**).

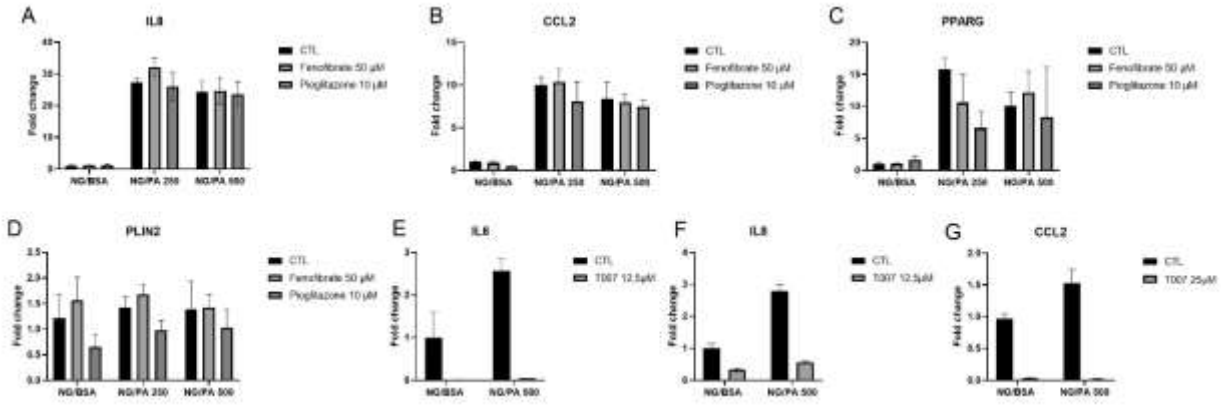


Fig. 8. *Relative expression of pro-inflammatory and lipid metabolism related genes from primary human monocyte culture in the presence of different PPAR agonists/antagonists.*

A–D) *RT-qPCR mRNA expression relative to RPS26 mRNA of A) IL8, B) CCL2, C) PPARG and D) PLIN2 mRNA expression normalized with S26 mRNA of primary human monocyte culture in different medium conditions in the presence of Fenofibrate 50 µM (Medium grey), Pioglitazone 10 µM (Dark grey) and Pioglitazone 50 µM (Light grey).*

E-G) *RT-qPCR mRNA expression relative to RPS26 mRNA of E) IL–6 and F) IL-8 and G) CCL2 mRNA expression normalized with S26 mRNA of primary human monocyte culture in different medium conditions in the presence of T007092 12.5 µM or 25 µM*

DISCUSSION

T2DM plasma induce upregulation of pro-inflammatory and lipid metabolism related genes.

We hypothesize that the abnormal dyslipidemic retinal environment in DR allows the differentiation of infiltrating Mo into pro-inflammatory Mϕ. In line with that we found MP (either activated microglia or extravasated Mϕ that entered to the retina due to impaired BRB) in the vicinity of microaneurysms and hemorrhages in retina samples of DR patients (**Fig. 2**

and Fig. 3). These MP are positive for PLIN2, a protein involved in lipid internalization and storage, that the host laboratory has shown to be largely overexpressed by MP differentiated in the presence of FFA. This suggest that the MP found in retina of DR patients have been in exposed to a lipid-rich environment. To confirm this hypothesis, we exposed naïve Mo to hi-plasma from T2DM with or without some degree of DR and showed an overexpression lipid storage and metabolism (*PLIN2, ACADVL, PDK4, PPARA*) related genes (**Fig. 4**) suggesting that a particular composition of lipids in the plasma of DR patients drives Mo to differentiate in MP adapted to lipid metabolism. This was further supported by the use of PA, an abundant FFA found elevated in the plasma of DR patient, which demonstrated the same overexpression in lipid storage and metabolism related genes. The switch in lipid metabolism was associated with a modified expression of pro-inflammatory cytokine (*IL-8* and *CCL2*) and molecules with vascular remodeling capacities (*VEGF* and *ANGPTL-4*) which suggest that the activation of MP may participate in DR pathological processes. Our results failed at demonstrating a correlation in cytokine production with the type of DR (PDR or NPDR). This is likely due to the size of the cohort and more patient may be needed to find differences. In lines with our findings correlation between plasma lipid levels and the presence of lipid laden Mϕ surrounding hard exudates (lipid rich deposit found in the retina of DR patients) has been reported suggesting that the differentiation of Mo in lipid rich environment may ultimately lead to the accumulation of lipid laden MP in DR retina. This accumulation of lipid laden MP is somehow similar to atherosclerosis (which is yet another vascular complication of T2DM) a situation where the role of PLIN2 in Mϕ has been partially described. The exact role of PLIN2 in DR still must be determined[49].

Surprisingly in our setting glucose did not induce Mo differentiation into pro-inflammatory Mϕ (unpublished data from host lab). This results is consistent with other studies showing that retinal cell react modestly to glucose when compared to FFA[50,51]. However, when PA-stimulated Mo were treated with insulin plus metformin, we observed an increase of IL6 secretion. All together these results suggest that not extracellular glucose concentration, but rather an increase of glucose uptake could worsen inflammatory profile of Mϕ.

PA induction of Mo to differentiate into pro-inflammatory Mϕ is associated with metabolic reprogramming.

O_2 consuming energetic pathway TCA/OXPHOS could be feed by various fuel source (glycolysis-derived pyruvate, acetyl-coA-derived from β-oxidation, or glutamine for a shunted

version of TCA). Extracellular acidification-linked to energy production could result from both aerobic glycolysis (1 glucose → 2 lactate + $2H^+$), or TCA (1 acetyl-CoA → 2 CO_2) in which CO_2 can be partially hydrated ($CO_2 + H_2O \rightarrow HCO_3^- + H^+$) resulting in extracellular acidification. Targeting different complexes of electron transport chain (ATP synthase, and complex I and III together) provide metabolic phenotype information.

Thus, higher basal ECAR and lower OCR values in PA-pretreated Mo indicated that the higher acidification in this condition is not linked to TCA/OXPHOS ATP production, because if this pathway were to be used, O_2 would also be needed. As another note, after Oligomycin and Rotenone/Antimycin injections we observed similar OCR levels in all groups, indicating a similar level of non-mitochondrial and H^+ leak linked to O_2 consumption. Thus, the lower basal OCR levels in PA-pretreated Mo is really due to a lower use of TCA/OXPHOS pathway for energy production.

Consequently, the increased ECAR observed in PA-pretreated group should be due to a higher aerobic glycolysis. Moreover, we found that after Oligomycin injection, ECAR rising is more elevated in the control group than in the PA-pretreated Mo group, indicating a lower glycolytic reserve for the PA-pretreated Mo, for which glycolysis may be already close to its maximal rate. These result fall in line with previous observations of host lab showing an increased concentration of extracellular lactate in the conditioned medium of PA treated Mo (unpublished data). To demonstrate a possible causative role of metabolic use of glucose in the presence of FFA in the production of inflammatory cytokine, we tested if a greater access of Mo to glucose (with the use of insulin or the insulin sensitizer metformin) would result in a greater pro-inflammatory differentiation of Mo. Our results show that Mo differentiation is independent of these stimulations. However, in our preliminary results a double stimulation by insulin and metformin increased IL-6 production suggesting that forced glucose usage may affect cytokine production under certain conditions. These results are in line with *Bunn et al.*[52], who showed that insulin in combination with PA induce the expression of pro-inflammatory genes in a THP-1 cell line, however these experiments have to be repeated (which was not possible due to the particular situation). There is a large number of studies which describe pro-inflammatory Mϕ relying more on aerobic glycolysis than the resting Mϕ[53], nevertheless the role of this metabolic switch is poorly known, though there is the hypothesis that aerobic glycolysis provides energy more quickly and will help with regulation of other metabolic pathways, such as lipid and amino acid biosynthesis[45]. Seahorse analysis of the insulin/metformin effect on O_2 consumption and

extracellular acidification have to be determined to definitely link cytokine production to aerobic glycolysis.

Otherwise beyond a similar metabolic profile to that of BSA, non-metabolizable analogues of PA, were also found unable to stimulate IL-6 secretion. Indicating that metabolization of PA is require for both metabolic switch and inflammatory polarization of Mϕ. Our data is consistent with recent demonstration that PA induced Mϕ polarization is not mediated by TLR4[45], and previous data indicating that metabolization of PA is needed for inflammatory polarization[52]

All together these results demonstrate that in the presence of PA the overexpression in lipid metabolism genes is not correlated to a shift from carbohydrate to lipidic fuel use. Quite the opposite PA-pretreated Mo rely more on aerobic glycolysis. Thus, the PA catabolic metabolites seem to have other implications in Mϕ polarization. When not used in TCA, acetyl-CoA could also be implicated in protein posttranslational acetylation. To note, histone acetylation in Mϕ have notably been associated with expression of inflammatory cytokines[54]

Regulation of Mo metabolism partially blunt the inflammatory response of Mo

RNAseq analysis of FFA effect on Mo performed by the host team demonstrate an upregulation of PPARγ, and thus suggest that the inflammation associated with the differentiation of Mo in a lipid rich environment may be regulated by regulators of PPAR activity. In a first set of experiment, our results demonstrate that drugs that activate/inhibit PPARs show mild efficacy at reducing IL-6 secretion. Consistently, we showed by q-PCR that both fenofibrate and pioglitazone (PPAR agonists) failed at blunting Mo activation, suggesting that the effect of fenofibrate observed in the ACCORD and FIELD trials is independent of a modulation of inflammation or an enhancement of lipid metabolism in Mϕ. However, in another set of experiment we demonstrate that T007097 a PPARγ antagonist, managed to reduce the overexpression of inflammatory genes such as *IL-6, IL-8* and *CCL2* in Mo seeded in the presence of PA. Nevertheless, T007097 also reduce the expression of these genes in the control condition, suggesting a possible off target effect. All together our results point out a complex role of PPARs in Mo differentiation, we ruled out the possibility that fenofibrate or pioglitazone may have a positive effect on Mo differentiation despite the reported positive effect of fenofibrate in DR[22,46] but surprisingly the use of antagonist of PPARγ may help at blunting the inflammatory response of Mo induced by lipids. These results have to be repeated and the effect of T007097 on Mo metabolism has to be determined using a detailed metabolic seahorse analysis. Finally, the use of inhibitors of lipid metabolism (Etomoxir and Triacsin C) also

resulted in a decreased production of IL-6 in PA treated Mo suggesting that intracellular lipid usage directly alter Mo differentiation.

Thus, we here demonstrate that in PA-treated Mo, intracellular use of lipid correlate to inflammatory response. However, the reduction of oxygen consumption (OCR) in PA-treated Mo suggests that PA catabolic metabolites do not participate in TCA/OXPHOS-linked ATP production. All together our results suggest that modifying Mo metabolism may be an attractive alternative to conventional corticosteroids to reduce inflammation during DR.

CONCLUSSION

DR is a neurodegenerative, microvascular and inflammatory complication of T2DM, where impairment to the BRB leads to extravasation of plasma proteins, lipids and leukocytes (including monocytes), Our data suggest that lipids found in the plasma of T2DM patients induce an upregulation of pro-inflammatory, lipid storage and metabolism-related genes in Mo. We showed that Mo activation is associated to a complex metabolic reprograming, in which genes implicated in the lipid metabolism PLIN2, ACADVL, PDK4 are upregulated together with inflammatory cytokines. Interestingly lipid-stimulated Mo does not increase their oxygen consumption, on the contrary elevated extracellular acidification suggest a strong aerobic glycolysis (also known as the Warburg effect). Finally, our results suggest that compounds inhibiting PPAR pathway as well as drug targeting key enzyme in lipid metabolism blunt Mo activation in a lipid rich environment.

To date the underlaying mechanism that leads Mo to differentiate into pro-inflammatory Mϕ in the presence of FFA are not well understood, more studies are needed in order to decipher these mechanisms. Understanding the lipids implication in metabolism changes of Mϕ could lead to the development of new therapeutic avenues for DR, and to metabolic diseases even beyond the retina.

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Annexes

Table 1. Primer sequences utilized for the qPCR

Gene	Forward primer	Reverse primer
ACADVL	CATACCCGTCCGTGCTCAA	GTCATTCTTGGCGGGATCGT
ANGPTL 4	CTCTCCGTACCCTTCTCCACT	AAACCACCAGCCTCCAGAGA G
CCL2	GCTATAGAAGAATCACCAGCAGCA A	TGGAATCCTGAACCCACTTCT G
IL-6	AGCCCACCGGGAACGAAAGA	TTCAGAGCCCGCAGCTTCCA
IL-8 (CXCL8)	GACCAAGGAAATCGGCCTC	GCCATACCTCTAGGCTGGCT
PDK4	CCTGTGAGACTCGCCAACA	CCACCAAATCCATCAGGCTCT
PLIN2	GCCTGTAAGGGGCTAGACAG	TCGTCACAGCATCTTTTGCC
PPARA	GGACAAGGCCTCAGGCTATC	CGCACTTGTCATACACCAGC
PPARG	GAGGGCGATCTTGACAGGAAA	GGGGTGATGTGTTTGAACTTG A
RPS26	TCGATGCCTATGTGCTTCCC	CAGCACCCGCAGGTCTAAAT
VEGF	GAGCTTCCTACAGCACAACA	GAGATCTGCAAGTACGTTCG