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Inflamación, degeneración y remodelación vascular en patologías retinianas

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CONTRIBUTIONS

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SUMMARY **pág.**

TABLE OF CONTENTS

ABBREVIATIONS

AMD	Age-related macular degeneration
BSA	Bovine serum albumin
CCL2	Chemokine ligand 2
CCR2	C-C chemokine receptor type 2
DME	Diabetic macular edema
DR	Diabetic retinopathy
FFA	Free fatty acids
GCL	Ganglion cell layer
GFAP	Glial fibrillary acidic protein
HbA1C	Glycosylated hemoglobin A1C
HG	High glucose
HUVEC	Human umbilical vein endothelial cells
Iba1	Ionized calcium binding adaptor molecule 1
IL-10	Interleukin 10
IL-1ß	Interleukin 1ß
IL-6	Interleukin 6
IL-8	Interleukin 8
MCP-1	Monocyte chemoattractant protein-1
Møs	Macrophages
Mos	Monocytes
MP	Mononuclear phagocytes
MP	Mononuclear-phagocyte
Myd88	Myeloid differentiation primary response 88
ND	Non-diabetic
NG	NormoGlucose
NPDR	Non-proliferative diabetic retinopathy
PA	Palmitate
PBMCs	Peripheral blood mononuclear cells
PDR	Proliferative diabetic retinopathy
PFA	Paraformaldehyde
PPARγ	Peroxisome proliferator-activated receptor-γ
ROS	Reactive oxygen species

T2DM	type 2 Diabetes Mellitus
TLR2	Toll-like receptor 2
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

INTRODUCTION

Diabetic Retinopathy

The retina is the most internal layer of the eye, is highly sensitive to light, it converts light signal into nerve impulse that will travel to the brain through the optical nerve producing the visual image. The light arrives to the cornea, crossing the pupil and the crystalline until it reaches the retina. A good communication and healthy layers are needed for a good vision.

The eye is an immunoprivileged organ, several diseases can affect directly or as a secondary cause; a complication of long-term diabetes is diabetic retinopathy (DR), the principal cause of blindness in adults, the damage of the ocular tissue occurs even in early stages of the disease [1]. DR is categorized based on the presence of ophthalmoscopically visible vascular lesions. Non-proliferative diabetic retinopathy (NPDR) is the earliest stage of DR and visual impairment occurs when diabetic macular edema (DME) happens. The progressive degeneration of retinal capillaries in diabetes causes retinal ischemia, which induces subsequent neovascular response if the ischemia is severe and characterizes proliferative diabetic retinopathy (PDR), which eventually leads to legal blindness if untreated [2]. The vision loss in diabetes is gradual, generally begins with a reduction in night vision or the loss of details in low-light condition [3].

Diabetic patients and animal models of type 2 Diabetes Mellitus (T2DM) demonstrate early retinal mononuclear phagocyte activation [4, 5] and intraocular robustly elevated levels of inflammatory cytokines (CCL2, IL-8 and IL-6 and VEGF) are invariably found in patients with DR [6]. Inflammatory mononuclear phagocytes (MP) found in retinas of DR patients originate from local proliferation/activation of retinal microglia (the resident macrophage of the retina) but also from blood-born inflammatory monocytes. Microglia and Müller cells are the first detector of metabolic changes in DR, once activated they migrate to the site of inflammation and produce pro-inflammatory cytokines, reactive oxygen species (ROS) and proteases, the prolonged activation causes the increase of glutamate and the consequent retinal neuronal cell death [7].

Hyperglycemia and dyslipidemia

DR patients are characterized by hyperglycemia and dyslipidemia and controlling one or the other factor limits the progression of DR. Dyslipidemia is characterized by alteration in the vesicular lipid transport but also by changes in the concentration of the bioavailability of free fatty acid (FFA) in blood. Among FFA, palmitate has been shown to be increased in diabetic

patients [8]; the elevation of plasma FFA plays a pivotal role in the development of type 2 diabetes, causing insulin resistance. Diabetes develops as pancreatic ß-cells are unable to produce enough insulin to compensate the resistance. During low energy intake the body use the fat reservoirs and FFA is released so other tissues can use it as fuel; if FFA are increase in plasma for several hours they cause insulin resistance [9]. Luong et al reported that palmitate is a potent inducer of ROS in pancreatic ß-cells, endothelial cells and adipocytes, among other cells [10].

FFA can induce insulin resistance in liver that cause an overproduction of glucose, and in skeletal muscle that produce an underutilization of glucose. Overweight people are likely to have an increase of FFA in plasma and therefore, an expected elevation in the level of glucose; however, only half of the obese people present abnormal glucose levels and some of them do not develop diabetes due to normal pancreatic ß-cells, FFA secret insulin and compensate the created resistance supporting a basal insulin secretion [9].

Previous work has shown that hyperglycemia sensitizes macrophages to FFA possibly by increasing their expression of TLR2 and TLR4 and subsequent myeloid differentiation primary response 88 (myd88) signaling [11]. When bovine retinal cells are stimulated with palmitate and high glucose a mitochondrial DNA damage is observed. And in human umbilical vein endothelial cells (HUVEC) cells the treatment with both increased ROS levels [10]. Taken together these suggest that dyslipidemia, may participate to monocyte activation and subsequent expression of cytokines which are found elevated in the vitreous of DR patients.

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Pro-inflammatory state

Pro-inflammatory cytokines play a key role in the pathogenesis of DR. When blocking IL-1ß (with anakinra) in a type 2 diabetes study, the levels of IL-6 and glycosylated hemoglobin A1C (HbA1C) are reduced while the C-peptide secretion is increased indicating that the function of ß-cells were improved. This could suggest a role of IL-1ß in modulating inflammation and metabolic dysregulation in diabetic patients [12]. The pro-inflammatory cytokine IL-8 and monocyte chemoattractant protein-1 (MCP-1) have been evaluated in PDR in vitreous fluid, the results showed higher levels in diabetic patients with a correlation with PDR activity; while the IL-10, an anti-inflammatory cytokine, is not increase in vitreous fluid but is certainly lower in serum of type 2 diabetes patients with PDR [13].

CCL2 expression in retina is low in physiological conditions but stress or damage can induce an increase in its expression. It is involved in the pathogenesis of wet AMD; the atrophic AMD produces CCL2, this infiltrate neurotoxic $CCR2^{+}$ monocytes-derived from circulating

blood into the retina, causing MP accumulation in the subretinal space and producing the PR degeneration. It has been described by the laboratory team, that activated macrophages (Møs) can release CCL2 in neuro-inflammatory conditions, also that $Iba1^+$ MP invade the subretinal space of transitional zone of atrophic zones in AMD [14]. Monocytes (Mos) will leave the bone marrow into the bloodstream, once they reach the infected tissue or site of inflammation, the differentiation towards Møs will take place. Møs can modify their response according to the stimuli presented in the environment, they are involved in inflammation and homeostasis to clear apoptotic cells or cell debris. In the lab team, using Iba1, they were able to determine in hyperglycemic rats the localization of microglia/macrophages in the retina and subretinal space between the outer segment and retinal pigmented epithelium cells; contrary as found in control rats, where the $Iba1^+$ cells were located in inner retina layer but no in the outer retina or subretinal space, showing that inflammation and accumulation of MP is involved in DR [2].

Ganglion cell layer neuronal death

Müller cells, astrocytes and microglia provides structural support and are involved in the maintaining of homeostasis in the retina. When inflammation occurs, these cells become activated and phagocytose apoptotic cells and cell debris. In DR the chronic gliosis cause an increase in the secretion of inflammatory cytokines, glutamate, ROS, nitrous oxide, proteases and VEGF, that lead to a vascular dysfunction and neuronal cell dysfunction and the exacerbation of retinal neuron cell death [7]. As Hu and collaborators wrote, interleukin-1ß (IL-1ß) can be produced by tissue Møs, Mos, Müller cells, astrocytes, and endothelial cells in some pathological situations [15]. Several studies mention that microglia, resident Møs and immune cells are involved in the pathogenesis of age-related macular degeneration (AMD) and DR; in AMD the PR cells are dying via IL-1ß produced by Møs, while in DR the ganglion cell layer and inner nuclear layer are dying via glutamate.

Tumor necrosis factor-α (TNF-α) is a potent mediator of leukostasis and this is induced by IL-1ß and VEGF in the retinal vasculature. It has been found increased in diabetic animals and patients, studies suggest that TNF-α can contribute to the blood-retinal barrier breakdown in DR by mediation of apoptosis and leukostasis in mice [16]. IL-ß and TNF-α play a major role in the recruitment and activation of monocytes in DR [7].

The loss of cells in the ganglion cell layer or reduction in the number of cell bodies in the retinal ganglion cell layer with reduction in the thickness of the inner and outer retina have been detected in diabetic retinopathy animal models. Several studies in diabetic animals have

found activated caspases in the retinas and TUNEL-positive neuronal cells, consistent with a role of apoptosis in the retinal neurons. Kern et al mentions that diabetes-induced deficits in RGC function might occur before morphological changes [3].

We hypothesize that in DR, accumulation of activated MP in the retina could be responsible for ganglion cell layer (GCL) neuronal loss.

The goal in our study is to determine:

1. If MP accumulate in the retina of patient
2. If MP activated by DR relevant stress produce cytokine found to be elevate in DR patient.
3. If MP found in diseased retina produce these cytokines.
4. If MP derived cytokine participate to GCL neuronal loss.

MATERIALS AND METHODS

Animals

Two months old male mice C57BL/6. Mice were sacrificed with CO_2 and cervical dislocation, the eyes were enucleated and the explant of the retinas was performed in PBS 1X. All animals were housed in a 12/12 h light/dark (100–500 lx) cycle with food and water available ad libitum.

Blood monocyte extraction, monocyte-retinal co-culture

Peripheral blood mononuclear cells (PBMCs) were obtained from heparinized venous blood from healthy volunteer individuals by centrifugation of whole blood on a Ficoll-Paque layer (GE Healthcare) and sorted with EasySep Human Monocyte Enrichment Cocktail (Stem Cells Technology). Mouse retinas were placed with the photoreceptors facing polycarbonate filters floating on DMEM, then the hMos supernatant was seeded on the retinas overnight, changed in the morning and in the afternoon (3 hours later).

Reverse transcription and real-time polymerase chain reaction (RT-PCR)

RT-PCR was used to quantify the pro-inflammatory genes secreted by the cells, Müller cells, and hMos. For the Müller cells, total RNA was isolated with NucleoSpin RNA XS kit (Macherey Nagel), following instructions from the manufacturer. The cells were in contact with normal glucose (NG) or high glucose (HG), and bovine serum albumin (BSA) or palmitate

(PA). The RT-PCR was performed, the primers used were S26, IL-8, IL-6, CCL2, and VEGF. For the hMos, total RNA was isolated with NucleoSpin RNA XS (Macherey Nagel), the cells were in contact with NG or HG, and BSA or PA. RT-PCR was realized, the primers used were S26, IL-8, IL-6, CCL2, and VEGF. Results were normalized by the expression S26. PCR reactions were performed in 45 cycles of 15s at 95ºC, 45s at 60ºC.

Multiplex

We performed a Bio-Plex™ 27-plex assay #m500kcaf0y following instructions from the manufacturer. We used supernatant of monocytes stimulated with palmitate (500 µM), BSA concentration was adjusted. We read it and the analysis was performed with a Bio-Plex® MAGPIX We quantify in a relative fluorescence level

RNAseq

For whole transcriptome analysis, healthy volunteer $CD14^+$ Mos were prepared as described above and stimulated for 18h with PBS-palmitate (500µM). After cell lysis, RNA was extracted using the Qiagen RNA Mini Kit with RNase (ribonuclease)–free DNase (deoxyribonuclease) I digestion. RNA sequencing libraries were constructed from 1 µg of total RNA using a modified TruSeq RNA Sample preparation kit protocol. Pass-filtered reads were mapped using STAR v2.6.1c and aligned to human reference genome GRCh38.92 [17]. The count table of the gene features was obtained using Feature Counts REF. Normalization, differential expression analysis and FPKM (fragments per kilobase of exon per million fragments mapped) values were computed using EdgeR [18]. Protein coding mRNAs with greater than 5 FPKMs in the palmitate group were selected.

Iba1 immunohistochemistry

Donor eyes with history of Diabetes and controls from USA (Kellogs eye hospital, Michigan) were used. Retinas were dissected, fixed in 4% PFA, and imbedded in paraffin. Immunohistochemistry was used to identify Mos and Møs in the subretinal and retinal area of human retinas. The dewaxing of the human eye retinas was performed with Salfesolv for 30 minutes. The rehydration was made with alcohol 100%, 90%, 70% and distilled water for 5 minutes each one. PBS/triton 0.5% was used for 5 minutes and PBS/horse serum (1:100) for 10 minutes. The first antibody was added overnight at room temperature, Iba1 (rabbit-anti-human 1:100). The correspondent secondary antibodies were used to reveal the primary antibodies,

Hoechst (Sigma-Aldrich, 1:1000) was used as a counterstain, for 1 hour at room temperature. Pictures of the retina were taken with a fluorescence microscope (Leica, DM 5500).

IL-8 and Iba1 immunohistochemistry.

Donor eyes with history of DR and controls from USA (Kellogs eye hospital, Michigan) were used. Retinas were dissected, fixed in 4% PFA, and imbedded in paraffin. Immunohistochemistry was used to identify MP in the subretinal and retinal areas of human retinas. The dewaxing of the human eye retinas was performed with Salfesolv for 30 minutes. The rehydration was made with alcohol 100%, 90%, 70% and distilled water for 5 minutes each one. PBS/triton 0.5% was used for 5 minutes and PBS/horse serum (1:100) for 10 minutes. The first antibody was added overnight at room temperature IL-8 (mouse-anti-human 1:100) and Iba1 (goat-anti-human 1:100). The correspondent secondary antibodies were used to reveal the primary antibodies, Hoechst (Sigma-Aldrich, 1:1000) was used as a counterstain, for 1 hour at room temperature. Pictures of the retina were taken with a fluorescence microscope (Leica, DM 5500).

Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay was used to identify the apoptotic cells, with detection of DNA breaks in the last phase of apoptosis. 4% paraformaldehyde (PFA) fixed mice retinas were pre-treated with frozen methanol for 20 minutes and then methanol/acetic acid (2:1) for 10 minutes. After washing with PBS 1X, the retinas were incubated with substrate (9/10) and enzyme (1/10) from the In-Situ Cell Death Detection Kit (Roche Diagnostics) overnight at 4ºC. Then, placed for 75 minutes at 37.5ºC. The reaction was stopped with PBS. Hoechst (Sigma-Aldrich, 1:1000) was used as a counterstain for 10 minutes.

RESULTS

To quantify the distribution of MP in the retina of diabetic retinopathy patients, we stained donor eyes with pan-marker Iba1 (Fig 1A, arrows). Iba1$^+$ cells were significantly increased in DR donors when compared to non-diabetic (ND) or diabetic (T2DM) without a DR diagnosis in central retina (Fig 1B) and peripheral retina (Fig 1C). There was no significant increase in Iba1$^+$ cells in the subretinal space (Table 1). We thus show that Iba1$^+$ MP are increased specifically in eyes with DR and the accumulation is mainly in the peripheral space of the inner retina.

Central retina			
ND		**DR**	
ID patient	**No. of cells (cm)**	ID patient	**No. of cells (cm)**
5761	0,000	2564	0,000
5761	0,000	2564	2,274
2432	0,000	2323	0,000
2432	1,911	2323	0,000
2399	0,000	2454	0,000
2399	0,000	2454	0,000

Peripheral retina			
ND		**DR**	
ID patient	**No. of cells (cm)**	ID patient	**No. of cells (cm)**
5761	0,000	3469	0,000
5761	0,000	3469	0,000
2391	0,000	2319	0,000
2391	0,000	2319	0,000
1087	0,000	1066	0,000
1087	0,000	1066	0,000

Table 1. Quantification of Iba1$^+$ cells in central and peripheral retina of non-diabetic (ND) and diabetic retinopathy (DR) donors. The results show the counting in subretinal space, given by cm in the retina, the counting was performed in two different slides from the same patient (ID attached).

Several studies suggest that inflammatory processes play an important role in the development of DR. CCL2, IL-8 and IL-6 and VEGF are invariably found significantly elevated in cohorts of patients with DR [19]. In addition other cytokines with a potential neurotoxic activity such as IL1-ß and TNF-α have been detected in vitreous samples of eyes with DR [20]. To determine if DR relevant-stress could trigger a pro-inflammatory response related with the development of DR we stimulate Mos with high glucose (HG) and palmitate (PA). NormoGlucose (NG) conditions were set to 5mM glucose (1g/dL). The high glucose concentration was 25 mM, equivalent to 4,5 g/dL, a blood glucose concentration observed in extreme cases of uncontrolled diabetes. Osmolarity was controlled in NG and HG medium by the addition of mannitol. Palmitate concentration was set to 500 µM. Palmitate dilution was achieved in the presence of BSA and ethanol. All medium was adjusted for BSA and ethanol concentration. Our results show a slight increase in the expression of pro-inflammatory

cytokines, CCL2, IL-6 and IL-8 when stimulated with HG in comparison to NG (compare NG/BSA to HG/BSA, Fig 2) while no regulation of VEGF was observed in these conditions. In sharp contrast, palmitate significantly increased the expression of CCL2, IL-6 and IL-8 in NG and PA conditions after 18 hours, with a maximum stimulation observed in the HG group (Fig 2). This evidence suggests palmitate and to a lesser extent glucose can induce a pro-inflammatory state with the production of inflammatory cytokine observed in diabetic retinopathy patients *in vivo*.

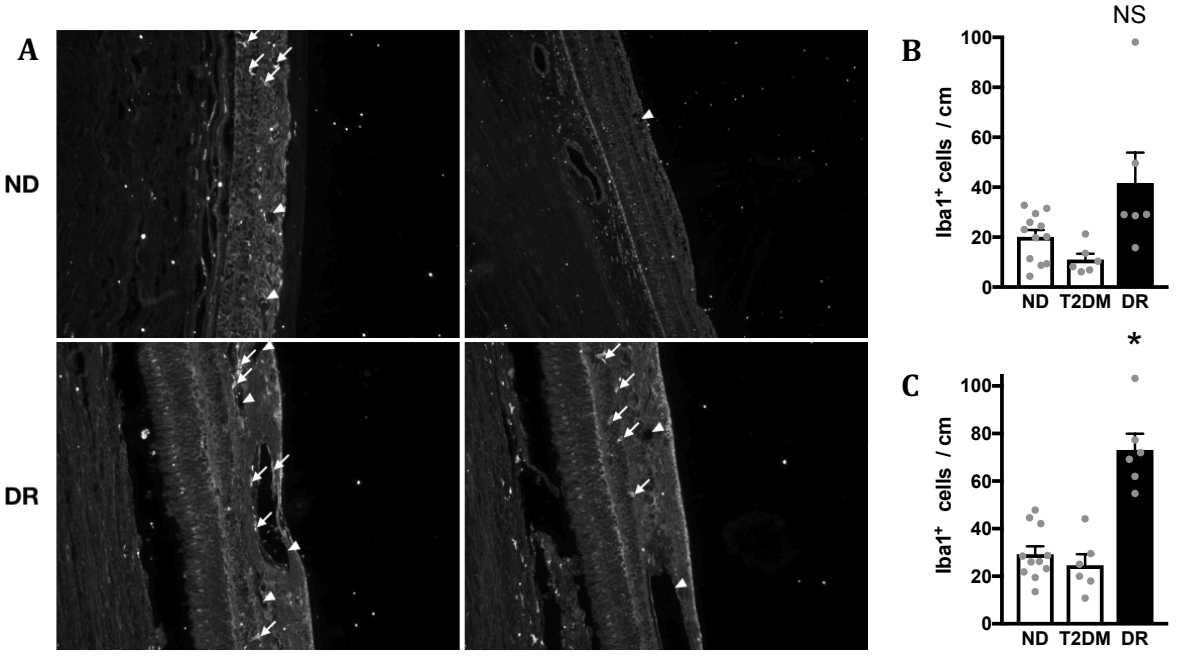

FIGURE 1. Localization of **(A)** Iba1$^+$ cells immunohistochemistry in non-diabetic (ND) and diabetic retinopathy (DR) donors. Arrows show Iba1$^+$ cells, head arrows show vessels. Two representative images from each group. Counting of Iba1$^+$ cells in **(B)** central retina and **(C)** peripheral retina of non-diabetic (ND), diabetic (T2DM) and diabetic retinopathy (DR) donors. *p=0,0038, ANOVA, Kruskal-Wallis followed by d

To confirm that palmitate is able to upregulate the secretion of the pro-inflammatory cytokine with normal or high glucose we performed a Luminex assay with Mos at 42 h (Fig 3). All inflammatory cytokines and the VEGF protein were increased with PA in NG or HG conditions while glucose very modestly increased the concentrations of these cytokines. IL-8 concentration in the supernatant of activated MP were extremely elevated (within ng/ml) making IL-8 a potential bio-marker of DR stress macrophages. To ensure that IL-8 was among the highest expressed and highest regulated gene, we RNAseq monocytes treated with palmitate in a normal glucose condition and plot all expressed genes (the normalized expression (FPKM)

>5) as a function of their regulation (FC) and expression level (FPKM) (Fig 4). Our results show an upregulation of the genes related with inflammation in DR such as IL-1ß and CCL2; and we confirmed that the RNA from IL-8 is among the most regulated and most expressed when stimulated with palmitate (Fig 4).

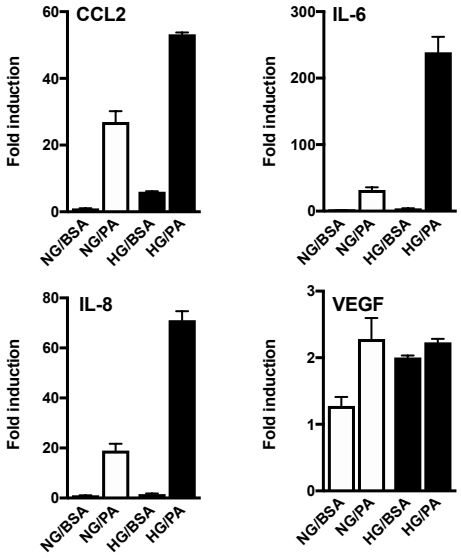

FIGURE 2. Gene mRNA expression in monocytes stimulated with normal glucose (NG) or high glucose (HG) with bovine serum albumin (BSA) or palmitate (PA) for 18 h, as determined by qPCR using S26 mRNA as reference. The expression of each gene after palmitate treatment is indicated. Shown is the ± SEM of three independent experiments.

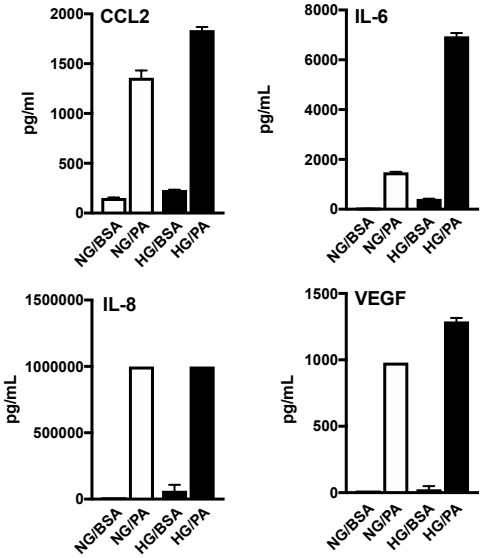

FIGURE 3. Quantification of interleukins CCL2, IL-6, IL-8 and VEGF. Monocytes stimulated with normal glucose (NG) or high glucose (HG) with bovine serum albumin (BSA) or palmitate (PA) for 42 h determined by Luminex.

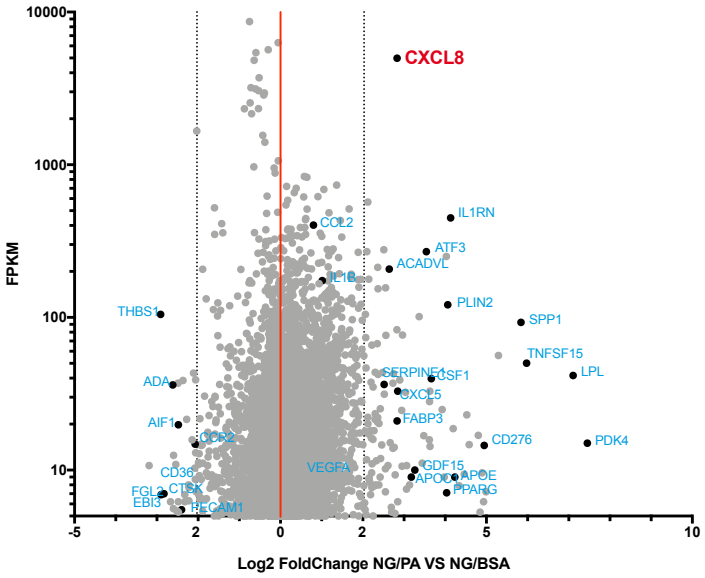

FIGURE 4. Most expressed interleukins and proteins after stimulation of macrophages with bovine serum albumin (BSA) and palmitate (PA) in a condition of normal glucose (NG), determined by RNAseq.

In DR the pro-inflammatory interleukins are increased. As we observed in qPCR, Luminex and RNAseq IL-8 was overexpressed, we therefore, analyzed central and peripheral retinas of non-diabetic (ND) and diabetic retinopathy (DR) donors. The localization of Iba1$^+$ MP was increased in central and peripheral retinas of the DR donors (expressed as Iba1$^+$ cells/cm; Fig 5). Immunohistochemistry shows the double labelling of the retinas with IL-8, numerous Iba1$^+$ cells also were IL-8$^+$ (Fig 5A, arrows; expressed as Iba1$^+$/IL-8$^+$ cells/cm). We show that Iba1$^+$/IL-8$^+$ Mos are increased in the retina of DR eyes compared with the ND individuals, in central retina (Fig 5C) Iba1$^+$/IL-8$^+$ cells are slightly increased compared with peripheral retina (Fig 5D).

Several reports of the lab team have shown that IL-1ß is secreted by MP, is found in increased levels and leads to severe cone segment degeneration [15, 21] and has been described as promoter chemokine expression during damage in AMD [22]. To confirm that palmitate is able to increase the secretion of IL-1ß with normal or high glucose we performed a Luminex assay with Mos at 18h (Fig 6A) and 42 h (Fig 6B) showing a significant increase of this cytokine. TNF-α is a potent mediator of leukostasis and also mediates the apoptosis of retinal neurons and vascular endothelial cells; Huang et al, demonstrated that TNFα is critical for the development of DR complications [16]; here we also confirmed that palmitate can induce an increased response of TNFα at 18h (Fig 6A) and at 42 h (Fig 6B). In IL-1ß and TNFα the maximum expression of cytokines was observed at 18 h after the stimulation (Fig 6A).

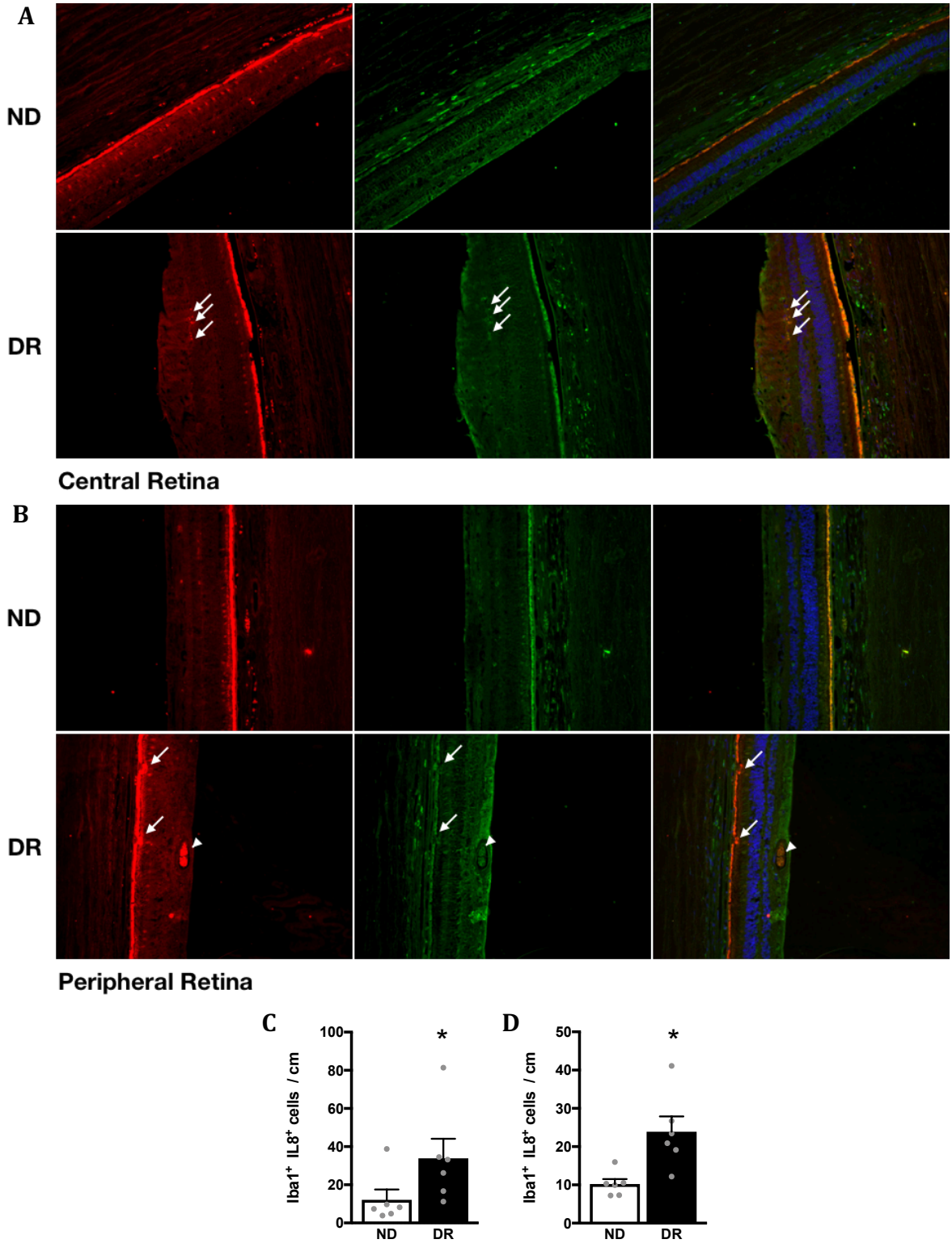

FIGURE 5. IL-8 (red staining) and Iba1 (green staining) on **(A)** central and **(B)** peripheral human retina of non-diabetic (ND) and diabetic retinopathy (DR) donors. Arrows show Iba1^{+}/IL-8^{+} cells, head arrows show vessels in the slides. Quantification of Iba1^{+}/IL-8^{+} cells in **(C)** central and **(D)** peripheral human retina of non-diabetic (ND) and diabetic retinopathy (DR) donors. *$p<0.05$, Mann and Whitney.

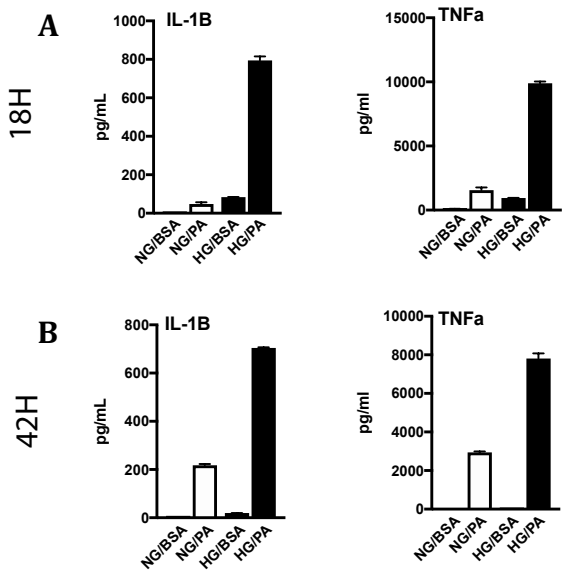

FIGURA 6. Quantification of interleukins IL-1ß and TNFα. Monocytes were stimulated with normal glucose (NG) or high glucose (HG) and with bovine serum albumin (BSA) or palmitate (PA) for **(A)** 18 h or **(B)** 42 h, determined by Luminex.

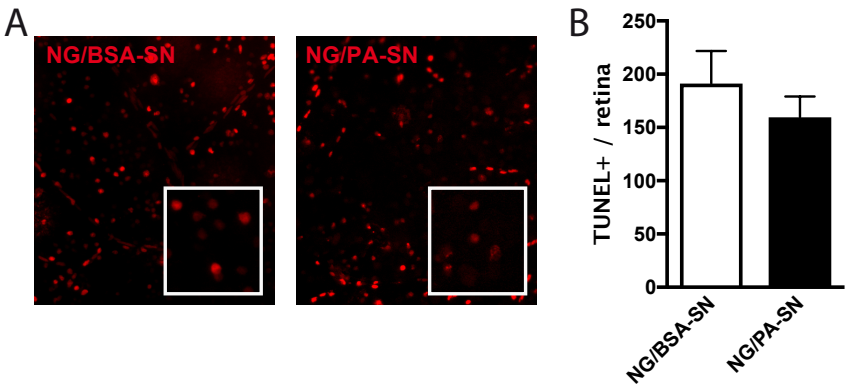

FIGURE 7. TUNEL stained retinal-flatmounts of **(A)** bovine serum albumin with supernatant of monocytes in normal glucose (NG/BSA-SN) and **(B)** palmitate with supernatant of monocytes in normal glucose (NG/PA-SN) treated for 12 hours. **(C)** TUNEL positive ganglion nuclei in NG/BSA-SN and NG/PA-SN treated for 12 hours.

The metabolic alterations associated with diabetes cause the Müller glial cells to upregulate the glial fibrillary acidic protein (GFAP), the GFAP activate the microglia, which are the resident immune cells of the retina, and start the production of pro-inflammatory chemokines. The activated pathways increase the production of ROS, pro-inflammatory and angiogenesis mediators that lead to neuro-glial neurodegeneration and vascular dysfunction. As mention by Rübsam et al, the retinal ganglion cells are the earliest cells affected in DR and have the highest rate of apoptosis [14]. We tested mice retinas with supernatant of Mos after stimulation with palmitate in a normal glucose condition for 12 h. To quantify cell loss in the

GCL after stimulation with supernatant we performed TUNEL stained retinal flatmounts of NG/BSA-SN and NG/PA-SN (Fig 7A, inset) and count the number of TUNEL positive cells in the GCL. We did not observe a difference between the number of dying cells in the GCL between these two conditions (Fig 7B).

DISCUSSION

MP are involved in the initiation and resolution of inflammatory processes; a deregulation or overstimulation and consequent activation can lead to chronic inflammatory diseases. Iba1 is a pan-MP marker, immunohistochemistry on ND and DR paraffin donor sections confirmed that MP accumulate in the retina in DR and that the subretinal cell layer is exempt of MP in healthy individuals (Fig 1A). Counting of $Iba1^{+}$ cells showed an increase in the presence of MP on peripheral retinas (Fig 1C) compared to central retinas (Fig 1B) in DR donors. MP are recruited in DR due to the pro-inflammatory state and we show that MP are highly increased in the retinal space.

Several inflammatory cytokines have been reported in NPDR and PDR patients. The cytokines are produced by microglia, endothelial cells, macroglia and even neurons, and are related with the progression of the disease, contributing to the cell death in the retina. Some of these cytokines are also thought to participate in angiogenesis [7]. The results from the qPCR (Figure 2) show that different pro-inflammatory cytokines (IL-6, IL-8 and CCL2) and VEGF that are related to DR, are increased after stimulation with palmitate.

As mention by Boden and Laakso, the elevation of free-fatty acids (FFA) plays an important role in the development of type 2 diabetes, causing insulin resistance [9]. Palmitate is one of the most abundant FFA in plasma; in individuals with type 2 diabetes, palmitate concentration in plasma is highly increased compared to healthy subjects [10]. The palmitate is able to induce a pro-inflammatory response by itself regardless of the normal or high glucose environment, but the response is enhanced when having a high glucose condition. After 42 h we observed that the inflammatory cytokines tested were highly increased after stimulation with palmitate (Fig 3). These results support that this pro-inflammatory response is involved with the recruitment of inflammatory cells that include Mos and Møs from blood stream. We tested the VEGF involved in vasculogenesis and neoangiogenesis, the cause of the complication of macular edema in DR; the results show that VEGF can be increased by palmitate or high glucose stimulation in a similar level.

The determination of a pro-inflammatory panel after monocytes stimulation with palmitate was realized with RNAseq showing an increase in pro-inflammatory cytokines and interestingly an overexpression in IL-8 (Fig 4). All these results together, lead us to investigate if IL-8 expression was also increased in DR human retinas. Immunohistochemistry using IL-8 show the increase in IL-8^{+} cells in DR donors compared with ND (Fig 5A, 5B). While the quantification of the IL-8^{+}/Iba1^{+} cells determined a higher expression in the central retina (Fig 5C) compared with the peripheral retina (Fig 5D). The inflammatory cytokines result in the development of new vessels in PDR, while VEGF itself serves as a pro-inflammatory molecule. IL-8 is a potent chemoattractant of inflammatory cells, meaning an active recruitment and accumulation that is involved in the development of DR.

IL-1ß and TNFα has been studied due to the strong relation with DR complications development. Our Luminex results showed an increase of the expression of the cytokines in monocytes after 18 h (Fig 6A) of stimulation with palmitate, these levels were slightly decrease after 42 h (Fig 6B) but maintained high, which can suggest that palmitate is closely involved in the generation of the pro-inflammatory response in DR as several inflammatory cytokines such as IL-1ß, TNFα, IL-8, IL-6, CCL2 and VEGF are increased or overexpressed. This evidence suggest that palmitate exposure has a bigger role than glucose in the development on later stages of DR and it may worsen diabetic complications.

Therefore, we wanted to evaluate the apoptosis caused by the infiltrated MP in the ganglion cell layer of mice retinas, we mounted TUNEL stained retinal flatmounts at 12 hours of exposure with the supernatant of monocytes with bovine serum albumin (BSA-SN) and with palmitate (PA-SN) (Figure 7A) to evaluate the effect of palmitate. PA-SN and BSA-SN flatmounts showed TUNEL-positive cells to a similar extent of induction of apoptosis (Figure 7B). According to our previous results with palmitate stimulation, the pro-inflammatory cytokines are increased and overexpressed creating a diabetic retinopathy-like type model which will produce the ganglion cell death, but TUNEL-assay results do not agree with the aforementioned. Also, the literature say that the loss of the neuronal cell by apoptosis should occur due to the presence of cytokines and growth factors; but Kern et al, mentions that in neuronal and capillary cells at any given time the number of apoptotic cells is very small, and that this may contribute to the slow onset and progression of the disease [3]. It can be possible that palmitate *in vitro* is not enough to activate and kill ganglion cells or that the time is too short to get an active response. Further studies are needed to get a conclusion from these results.

CONCLUSIONS

According to World Health Organization (WHO), the number of diabetic people has increased from 108 million in 1980 to 422 million in 2014. The prevalence of diabetes is rising, by 2040 the actual number is expecting to increase to 642 million people affected; is expected by 2030 to be the seventh cause of mortality in the world.

As mention by Zachary et. al., the principal treatment for macular edema is laser photocoagulation, the Early Treatment Diabetic Retinopathy Study showed a 50% reduction in vision loss; nevertheless, 12% can still lost some letters and having adverse consequences of leaking aneurisms, burns and scar expansion. Metabolic control included glycemic control, regulation of blood pressure and hyperlipidemia can decrease the risk of development of DR. Photocoagulation is effective at slowing the progression of DR but does not restore lost vision and there are patients that do not respond to this treatment, in these cases other intravitreal therapies are used, like the corticosteroids and anti-VEGF agents. The corticosteroids decrease inflammation, retinal capillary permeability and macular edema; while the anti-VEGF agents reduce the permeability and decrease the vascular proliferation of neovascularization. Most of these drugs present side effects due to the delivery, intravitreal injections, that includes glaucoma, retinal detachment, hemorrhage, cataract, pseudo-endophthalmitis and endophthalmitis. As a consequence, there is an increasing interest in the development of new topically administered therapies [23, 24].

In the other hand, as mention by Harris and collaborators, from a societal perspective, the costs of caring for patients with PDR (292 USD) are four times greater than managing patients with NPDR (1,207 USD) [25]; preventing the disease or detecting in early stages and being able to control it from the beginning will mean a diminish in cost and will increase the quality in life in diabetic patients. Added to this, a blood glucose and hypertension control are required to decrease the risk of vision loss.

Studies in diabetic patients and diabetic animal models have shown that diabetic environment cause an increase in the inflammatory cytokines and growth factors implicated in the pathogenesis of DR. In this work we show that palmitate, a FFA in blood, is able to promote the pro-inflammatory response as we observe with the increase in expression of inflammatory cytokines and the overexpression of IL-8. DR individuals are characterized by dyslipidemia, which is involved in this inflammatory response and development of DR.

Additional studies are still needed to find a better way to handling of the FFA in DR patients but decreasing the FFA in plasma levels can be a therapeutical approach to stop the development of the complications of DR, for this purpose the peroxisome proliferator-activated

receptor-γ (PPARγ) have been suggested as an endogenous inhibitor of insulin resistance; in macrophages PPARγ inactivation leads to glucose intolerance and impaired insulin function. Using a PPARγ agonist as a therapeutical approach may be a way of control the FFA due to its hypoglycemic effects in diabetic patients [26]. Work still needed to understand the molecular mechanism of the inflammatory response due to the FFA in DR.

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RESUME

Diabetic retinopathy (DR) is the principal cause of blindness in adults, is associated with ocular inflammation and the recruitment of mononuclear phagocytes (MP). In this study we show that DR is related with an increased retinal expression of several pro-inflammatory cytokines that are involved in the later complications and consequent development of DR. Inflammatory processes play a major role in DR, with immunohistochemistry we localized $Iba1^+$ cells into the retinal layer, showing an increase in the recruitment of inflammatory MP in DR individuals. To determine whether a DR relevant-stress can cause this inflammatory state, we stimulate monocytes with palmitate and quantify some cytokines related with DR, we find IL-6, IL-8, CCL2 and VEGF increased after stimulation. Then, with the use of Luminex and RNAseq we demonstrate that all inflammatory cytokines are increased and that IL-8 is overexpressed in monocytes after stimulation, these results suggest that palmitate can induce a pro-inflammatory state as observed in DR. We studied the expression of $IL\text{-}8^+$ cells in human retinas, findings show co-expression of $IL\text{-}8^+$ cells where $Iba1^+$ MP cells; also, the expression of $IL\text{-}8^+$ cells were increased in DR donors when compared with ND patients. As IL-1ß and TNF-α have been reported increased in DR individuals and are involved in neurotoxic activity we quantify the expression in monocytes after palmitate stimulation, results show highly increased expression of both cytokines. In DR the retinal ganglion cells are the first line of cells affected, we performed a TUNEL assay to determine the possible effect of palmitate stimulation in monocytes but showed no difference in apoptosis rate. We conclude that palmitate is able to induce a pro-inflammatory state similar as the DR response *in vivo*.