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Vitreous from patients with proliferative diabetic retinopathy induced changes in neutrophil activation markers

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Purpose: Diabetic retinopathy (DR), a microangiopathic complication of diabetes mellitus, is a leading cause of vision loss in working-age adults and older individuals. While the etiology of DR is not fully understood, it is strongly linked to systemic and local inflammation. Systemic immune-inflammation indices, such as the platelet-to-lymphocyte ratio, neutrophil-to-lymphocyte ratio, and monocyte-to-lymphocyte ratio, are useful predictors of diabetes mellitus–related diseases and inflammatory complications. In addition to systemic markers, local inflammatory molecules and immune cells, particularly neutrophils and their associated inflammatory mechanisms, play crucial roles in DR pathogenesis. Cumulative evidence indicates a concentration of inflammatory mediators in the vitreous humor, making its analysis a valuable tool for investigating retinal complications. This study aimed to identify differential cytokine expression in the vitreous humor of patients with diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) and to determine the impact of these vitreous samples on neutrophil activation.

Methods: Vitreous samples were collected during vitrectomy from patients with DME (n = 8), patients with PDR (n = 15), and surrogate controls (n = 8; rhegmatogenous retinal detachment, n = 5; macular hole, n = 3). Undiluted vitreous samples from the central vitreous cavity were analyzed individually via an angiogenic cytokine protein array at a concentration of 250 mg/ml of vitreous proteins. Cytokine levels were normalized to those of surrogate controls, and fold changes were calculated. For in vitro neutrophil stimulation, peripheral blood was incubated with diluted vitreous from different conditions, and neutrophil activation markers (CD15, CD11b, and CD66b) were assessed via flow cytometry.

Results: The study revealed increased neutrophil-to-lymphocyte ratio and monocyte-to-lymphocyte ratio values in patients with PDR and DME compared with controls ($p < 0.05$). Compared with those from controls, the vitreous from patients with PDR presented a twofold increase in the expression of the inflammatory cytokines CCL2, CXCL5, and angiogenin. Notably, compared with the control vitreous humor, the PDR vitreous humor significantly downregulated the neutrophil activation markers CD11b and CD15 ($p < 0.05$), while CD66b expression remained unchanged ($p > 0.05$). The DME vitreous did not significantly change any of the analyzed neutrophil activation markers.

Conclusions: This study highlights the importance of inflammation and its components in the pathophysiology and progression of DR and suggests that CCL2, CXCL5, and angiogenin are potential therapeutic targets for PDR. Our results also suggest that vitreous fluid from patients with PDR contains immunosuppressive or exhaustion-inducing factors that may alter neutrophil function and inflammation in DR.

Diabetes mellitus (DM) is a chronic, highly prevalent worldwide disease characterized by sustained high levels of glucose in the blood. Diabetic retinopathy (DR), nephropathy, and neuropathy are considered microangiopathic complications of DM, and the former is recognized as the main cause of visual loss in working-age adults and older people. It is estimated that 160 million people will have DR by 2045 worldwide [1]. The etiology of DR is still a matter of active research; however, it is widely accepted that systemic and local inflammation are closely related. Diabetic macular edema (DME) is a complication that may occur at any stage of DR. It is characterized by thickening of the retina involving the fovea and is caused by abnormal fluid accumulation. DME is the leading cause of vision loss in patients with DR [2]. We previously described that patients with DR presented elevated serum proinflammatory cytokines such as interleukin (IL) 6 and tumor necrosis factor α (TNFα), which are positively correlated with clinical parameters, glycated hemoglobin, body mass index, and serum creatinine [3]. Moreover, a significant association of the systemic immune-inflammation index, platelet-to-lymphocyte ratio [4], and neutrophil-to-lymphocyte ratio [5] with the presence of DR and DME has been established [6]. Additionally, high systemic immune-inflammation index values predict renal

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failure, peripheral arterial disease, and hospitalizations in patients with DR [7]. In addition to systemic markers, local inflammatory molecules play crucial roles in DR. Cumulative evidence indicates that many of these mediators are concentrated in the vitreous humor—a gel-like substance that fills the posterior chamber of the eye. The vitreous, which is primarily composed of water, hyaluronic acid, electrolytes, and proteins, serves vital metabolic and structural functions. Its transparency and absence of blood vessels facilitate light transmission to the retina, while its antioxidant properties protect the lens from oxidation through ascorbate-dependent mechanisms. Moreover, the vitreous acts as a reservoir for nutrients, including glucose, which diffuses to the retina [8]. Modifications in the microenvironment of the vitreous caused by the presence of nonvitreous proteins have been associated with the development of pathologies. For example, the levels of proinflammatory cytokines, such as IL-33, CXCL5, IL-7, and CD5, are elevated in traumatic vitreoretinopathy [9]. The presence of TNFα and IL-1β in the vitreous induces C16 ceramide production, contributing to the pathogenesis of DR [10]. Elevated selenium levels have been detected in vitreous samples from patients with proliferative diabetic retinopathy (PDR) [11]. Additionally, metabolomic studies have revealed altered levels of metabolites, such as phenylacetyl glutamine, pantothenate, and tyrosine, in the vitreous of patients with PDR [12]. It has also been reported that there is a decrease in adiponectin and an increase in interferon γ and TNFα levels in vitreous samples from patients with DR [13]. Proteomic analyses have revealed increased concentrations of apolipoproteins and proteins from the coagulation and complement cascades in PDR and DME. Similarly, the levels of inflammatory molecules, including CCL2, pro–IL-1β, IL-1β, and transforming growth factor β, are elevated in age-related macular degeneration [14]. Proliferative vitreoretinopathy is associated with increased levels of proteins such as kininogen 1, insulin-like growth factor–binding protein 6, p53, complement factors, tubulin, opticin, interferon γ, IL-6, IL-8, TNFα, CXCL10, and CCL3 [14]. Therefore, the study of vitreous samples is valuable for investigating localized complications of systemic diseases such as DR and DME.

Neutrophils are the most abundant leukocytes in the blood and the primary defense cells of the innate immune system that respond to harmful stimuli by increasing reactive oxygen species (ROS) production, releasing neutrophil extracellular traps (NETs), undergoing degranulation, and increasing phagocytosis. In the context of diabetes, elevated glucose levels act as a harmful stimulus, activating neutrophils and triggering increased ROS production and NET release [15]. However, despite this heightened activation, the ability of neutrophils to eliminate pathogens is impaired, as high glucose levels inhibit their phagocytic capacity [16]. Moreover, the release of NETs and their molecular components is associated with diseases such as DM, sepsis, and cardiovascular, renal, and liver diseases, among many others [17]. We recently reported that spontaneous NET release from freshly isolated peripheral blood neutrophils serves as a marker associated with inflammation, hyperglycemia, and renal disease in individuals with DR. Additionally, the expression of CD11b and CD66b neutrophil activation markers is positively correlated with DM duration [18]. These findings suggest that systemic inflammation provoked by neutrophils is associated with DR pathophysiology and its associated complications. For example, neutrophil dysregulation is associated with DM atherosclerosis in a ROS- and peptidyl deaminase 4–dependent manner [19]. In both nonproliferative DR and PDR, intricate mechanisms of activated neutrophils, such as the polyol pathway, leukocyte adhesion, the regulation of diverse RNAs, pyroptosis, and ferroptosis, among others, have been described [20], indicating the active function of neutrophils in DR. The aim of the present study was to identify the differential expression of cytokines in the vitreous between patients with DME and PDR and to determine whether the vitreous can modify neutrophil activation markers.

METHODS

Participants: This study included patients who underwent vitreoretinal surgery at the Institute of Ophthalmology Conde de Valenciana Foundation in Mexico City, Mexico, between March 2022 and August 2024. The vitreous samples were obtained from patients who underwent surgery for DME (n = 8), PDR (n = 15), and surrogate controls (n = 8), which included patients with rhegmatogenous retinal detachment (n = 5) and macular holes (n = 3). None of the patients received any anti–vascular endothelial growth factor (VEGF) treatment before vitreous collection. All participants were fully informed about the study and provided written informed consent voluntarily. The study adhered to the tenets of the Declaration of Helsinki. The protocol was reviewed and approved by the institutional review board and ethical committee (CEI-2022/03/03). The clinical data of all the enrolled patients were recorded. Patients with autoimmune diseases or a history of ocular surgeries, including intravitreal injections, were excluded from the study. Undiluted vitreous (UV) samples were collected from the central vitreous cavity of patients undergoing vitrectomy via pars plana with a 25-gauge vitrector. Briefly, a 5-ml sterile syringe was connected to the stopcock of the vitrector aspiration line. After a closed infusion tube was secured to the inferior

temporal sclera, 0.4 to 0.8 ml of undiluted core vitreous was aspirated into the syringe via active cutting in combination with syringe suction. The samples were then transferred to sterile 1.5-ml microfuge tubes and immediately stored at −80 °C for subsequent analysis.

Quantification of vitreous proteins: The total protein content of the collected UV samples was quantified via a modified Lowry protein assay kit (23,240; Thermo Scientific, Waltham, MA). Briefly, a standard curve was generated according to the manufacturer's protocol. Afterward, 50 µl of UV and standard dilutions were pipetted into a microplate well, and 200 µl of diluted Folin reagent was added. The samples and dye reagent were mixed and incubated at room temperature (RT, 25 °C) for 30 min in the dark. The absorbance was immediately measured at 595 nm in a plate spectrophotometer.

Angiogenic cytokine protein array: Human angiogenic protein arrays were performed according to the manufacturer's instructions (ab134000; Abcam, Cambridge, UK). Briefly, every membrane array was blocked for 30 min at RT. Afterward, the arrays were incubated overnight at 4 °C with shaking, containing 250 mg/ml vitreous protein. Following incubation, the arrays were washed three times with wash buffer I solution and twice with wash buffer II solution and incubated with biotinylated antibody cocktail for 2 h at RT with shaking. After washing, the arrays were incubated with horseradish peroxidase–conjugated streptavidin at RT for 2 h and then washed as described previously. The arrays were visualized with chemiluminescence buffer, and the signals were detected via G-BOX equipment and Gene Snap software version 7.12.06 (Syngene, Cambridge, UK). The analysis was performed via Gene Tools software (Syngene), and integrated density values for each spot were calculated by multiplying the area by the relative intensity. The negative controls were used to subtract background signals. The cytokine levels in the DME and PDR groups were normalized to those of the surrogate controls, and the fold change for each cytokine was calculated.

Neutrophil activation: Peripheral blood samples from healthy volunteers were collected in sodium citrate anticoagulant tubes, and 0.1 ml of complete blood was incubated for 1 h at 37 °C in 5% CO_2 with a 1:4 dilution of vitreous. The tubes were subsequently incubated for 30 min at 4 °C in darkness with a mixture of FITC-CD15, PE-CD11b, and BV450-CD66b antibodies (BD Biosciences, San Diego, CA). Next, a fixing and erythrocyte lysing solution (BD FACS Lysing; BD Biosciences) was added for 15 min at RT in the dark. A total of 1×10^4 cells from the polymorphonuclear population were acquired on a BD FACS Lyric flow cytometer (Biosciences BD). The median fluorescence index data were analyzed via FlowJo 10.0 v software (FlowJo LLC, Ashland, OR).

Statistics: The data were analyzed via one-way analysis of variance, followed by Bonferroni post hoc correction and, in some cases, the Mann-Whitney test. The results are presented as the mean ± standard error of the mean, with $p < 0.05$ considered statistically significant. All the statistical analyses were performed via GraphPad Prism (version 8.0.2; GraphPad Software, La Jolla, CA).

RESULTS

Demographic data and clinical data: The patients included in the present study presented similar demographic data. Although there were slight differences in the serum biochemistry levels of cholesterol, triglycerides, and creatinine among the groups, statistically significant differences between the groups were not found. Interestingly, the hematological inflammatory ratios, such as the neutrophil-to-lymphocyte ratio (NLR) and monocyte-to-lymphocyte ratio (MLR), differed between the DME and PDR groups and the control group ($p < 0.05$). Increased NLR and MLR values were found in patients with PDR. The demographic and clinical data are summarized in Table 1.

Quantification of cytokines in vitreous samples: Analysis of cytokine levels in vitreous samples revealed significant findings. Among all the cytokines measured, CCL2, CXCL5, and angiogenin were markedly elevated in the PDR group, showing more than a twofold increase compared with the surrogate control group. Interestingly, these same cytokines—CCL2, CXCL5, and angiogenin—were also upregulated in the DME group; however, the increase was less pronounced, remaining below a twofold increase. On the other hand, other proangiogenic cytokines, such as TIMP-1 and TIMP-2, leptin, VEGF-D, and CXCL1, are produced by at least one unit on the vitreous vein of patients with PDR; nevertheless, this elevation was not detected in DME and control samples (Table 2 and Figure 1).

Effects of vitreous samples on the activation markers of peripheral blood neutrophils: The incubation of vitreous samples from the different study groups of human neutrophils revealed distinct effects on the expression of activation marker membranes. Compared with those from the surrogate controls, those from the PDR group exhibited significant decreases ($p < 0.05$) in the expression of CD11b and CD15 activation markers, whereas CD66b expression remained unchanged ($p > 0.05$). In contrast, vitreous samples from the DME group did not significantly change ($p > 0.05$) the expression of CD11b, CD15, or CD66b activation markers compared with those from the surrogate controls (Figure 2).

Table 1. Demographic and clinical data of the subjects enrolled in the study.

Diabetes (DM)			
Demographics	Surrogate controls	Macular edema	Proliferative DR
n	8	8	15
Sex (F:M)	6/2	5/3	7/8
Age (±SD)	61.7(±4.3)	61.4(±6.0)	57.2(±11.5)
DM evolution (years)		13.8(±6.7)	19.4(±7.0)
Clinical biochemistry			
Cholesterol (mg/dl)	192.8 (±35.7)	185.3 (±35.3)	176 (±59)
Triglycerides (mg/dl)	128.9 (±28.9)	178.6 (±63.0)	210.5 (±52.6)
Creatinine (mg/dl)	0.74 (±0.14)	1.1 (±0.5)	1.6 (±0.6)
Hematological Inflammatory Ratios			
NLR	1.33	2.50 (*p=0.04)	3.60 (*p=0.03)
PLR	134	156 (p=0.28)	131 (p=0.46)
MLR	0.18	0.3 (*p=0.01)	0.36 (*p=0.01)

NLR, neutrophil-to-lymphocyte ratio; *PLR,* platelet-to-lymphocyte ratio; MLR, monocyte-to-lymphocyte ratio; (*p<0.05) p value versus surrogate controls

DISCUSSION

DR is a major complication of DM and is becoming increasingly prevalent worldwide. Growing evidence indicates that both systemic and localized inflammation play critical roles in the pathophysiology of DR. In this study, we analyzed the levels of angiogenic cytokines in the vitreous humor of patients with PDR and DME, as well as surrogate control subjects. Our findings revealed that the chemokines CCL2 and CXCL5, as well as angiogenin, were significantly upregulated in patients with DR compared with controls. Notably, these three cytokines were also elevated in patients with DME, although their levels were less pronounced than those in patients with DR.

CCL2, also known as monocyte chemoattractant protein 1, is a potent chemotactic factor for monocytes, macrophages, and T and natural killer lymphocytes. This O-glycosylated chemokine drives its activity through CCR2 and is produced by different cells, including endothelial, epithelial, smooth muscle, mesangial, and microglia, as well as fibroblasts, monocytes, macrophages, and astrocytes [21].

Table 2. Cytokine quantification* in vitreous from DME and PDR.

Cytokine	DME	PDR	Cytokine	DME	PDR
CCL2	0.23	8.15 ↑	*PDGF*	0.06	0.76
CXCL5	0.42	7.20 ↑	*TPO*	0.04	0.75
Angiogenin	0.67	3.80 ↑	*TGF-β*	0.01	0.69
TIMP1	0.15	1.99	*CCL5*	0.07	0.64
Leptin	0.09	1.65	*IGF1*	0.08	0.63
TIMP2	0.21	1.61	*VEGF*	0.06	0.63
VEGFD	0.06	1.15	*IL-8*	0.02	0.62
CXCL1	0.12	1.09	*PGF*	0.02	0.57
EGF	0.07	0.95	*IL-6*	0.00	0.20
IFN-γ	0.09	0.82	*bFGF*	0.00	0.07

*Results are expressed as a fold increase over surrogate controls. ↑ elevated cytokine levels, DME, diabetic macular edema; PDR, proliferative diabetic retinopathy; TIMP1, tissue inhibitor of metalloprotease 1; VEGF, vascular endothelial growth factor; EGF, epidermal growth factor; IFN-γ, interferon γ; PDGF, platelet-derived growth factor; TPO, thrombopoietin; TGF-β, transforming growth factor β; IGF1, insulin-like growth factor; PGF, placental growth factor; bFGF, basic fibroblast growth factor.

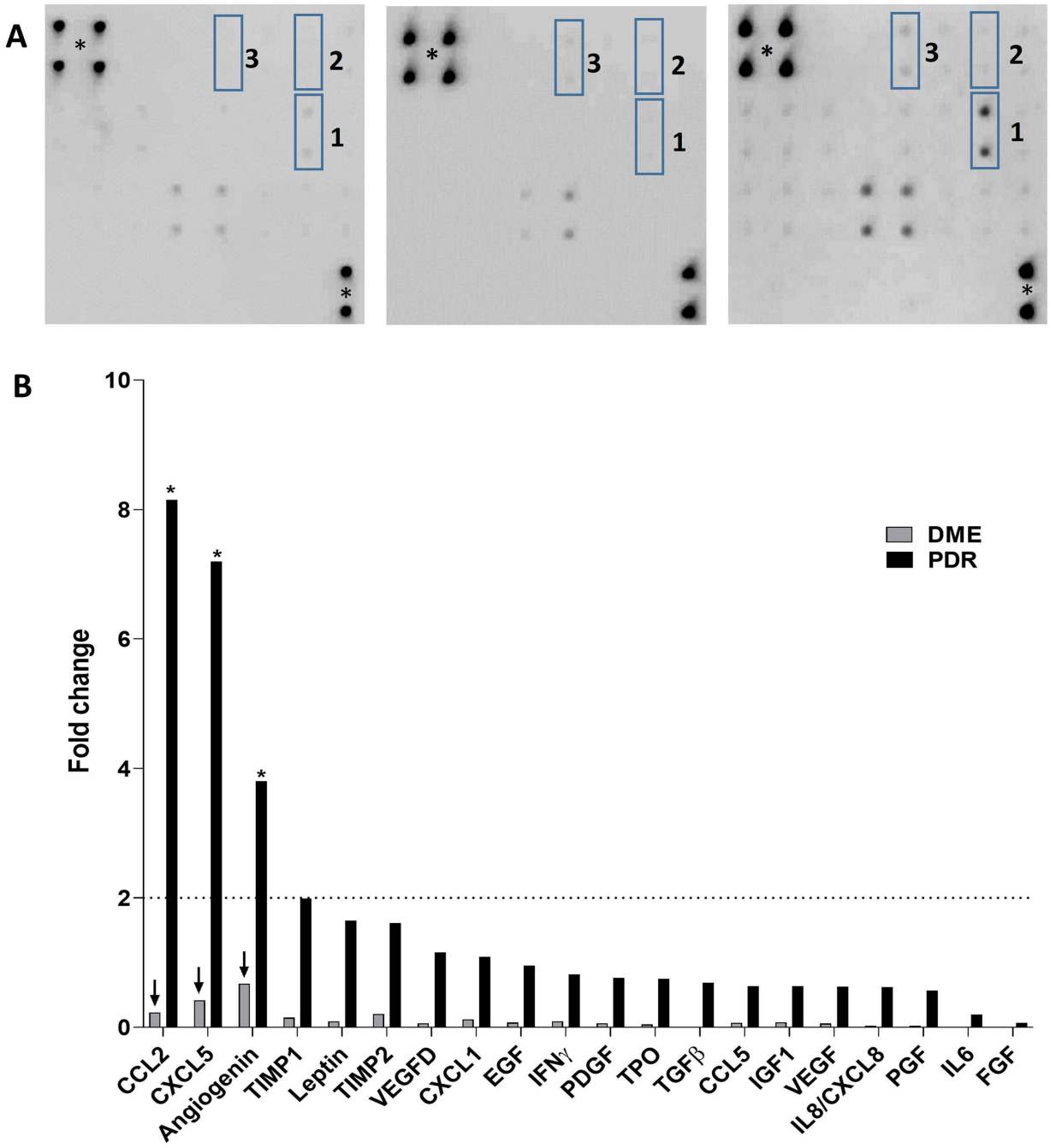

Figure 1. The vitreous of patients with proliferative diabetic retinopathy (PDR) and diabetic macular edema (DME) contains proteins that are significantly upregulated compared with control samples. **A**. Human angiogenic array membranes from surrogate controls (left membrane), diabetic macular edema (DME; middle membrane), and proliferative diabetic retinopathy (PDR; right membrane) vitreous samples. Numerals represent angiogenic proteins. 1. CCL2. 2. CXCL5. 3. Angiogenin. Asterisks indicate positive controls. **B**. Bar graphs showing the proteins that are upregulated in the vitreous from patients with DME (gray bars) and PDR (black bars). The data are presented in the order of change in protein expression over surrogate controls. Asterisks indicate the proteins whose expression is significantly upregulated twofold, as indicated by the dashed line. The vitreous of patients with PDR presented a twofold increase in CCL2, CXCL5, and angiogenin. *$p < 0.05$. **$p < 0.01$.

CCL2 augmentation in DR disrupts the blood-retinal barrier, increasing vascular permeability and facilitating leukocyte recruitment. High glucose levels stimulate CCL2 synthesis and secretion in retinal endothelial cells, increasing vascular permeability and highlighting the critical role of CCL2 in DR progression [22]. Our study revealed a significant increase in CCL2 concentrations in the vitreous of patients with PDR, with an increase of up to eightfold compared with that in controls. This finding suggests a localized inflammatory response distinct from that observed in DME. Our findings revealed persistently elevated CCL2 levels in patients with PDR who underwent repeat vitrectomies, which were associated with prolonged inflammatory processes and complications such as tractional retinal detachment [23]. Moreover, previous studies have reported increased CCL2 levels in both the aqueous humor [24] and vitreous of patients with PDR [25] compared with nondiabetic controls.

We observed a slight but nonsignificant increase (0.23-fold increase) in CCL2 levels in the vitreous humor of patients with DME compared with controls; interestingly, similar behavior was reported by Batsos et al. [26], who reported that CCL2 levels in patients with DME were elevated relative to those in controls, but the increase was not statistically significant. Additionally, inhibition of both the chemokine receptors CCR2 and CCR5 was associated with only a modest improvement in DME, suggesting that mechanisms other than CCL2-mediated inflammation are involved in this DM complication [27].

CXCL5, also known as epithelial-derived neutrophil-activating peptide 78, was first identified in activated

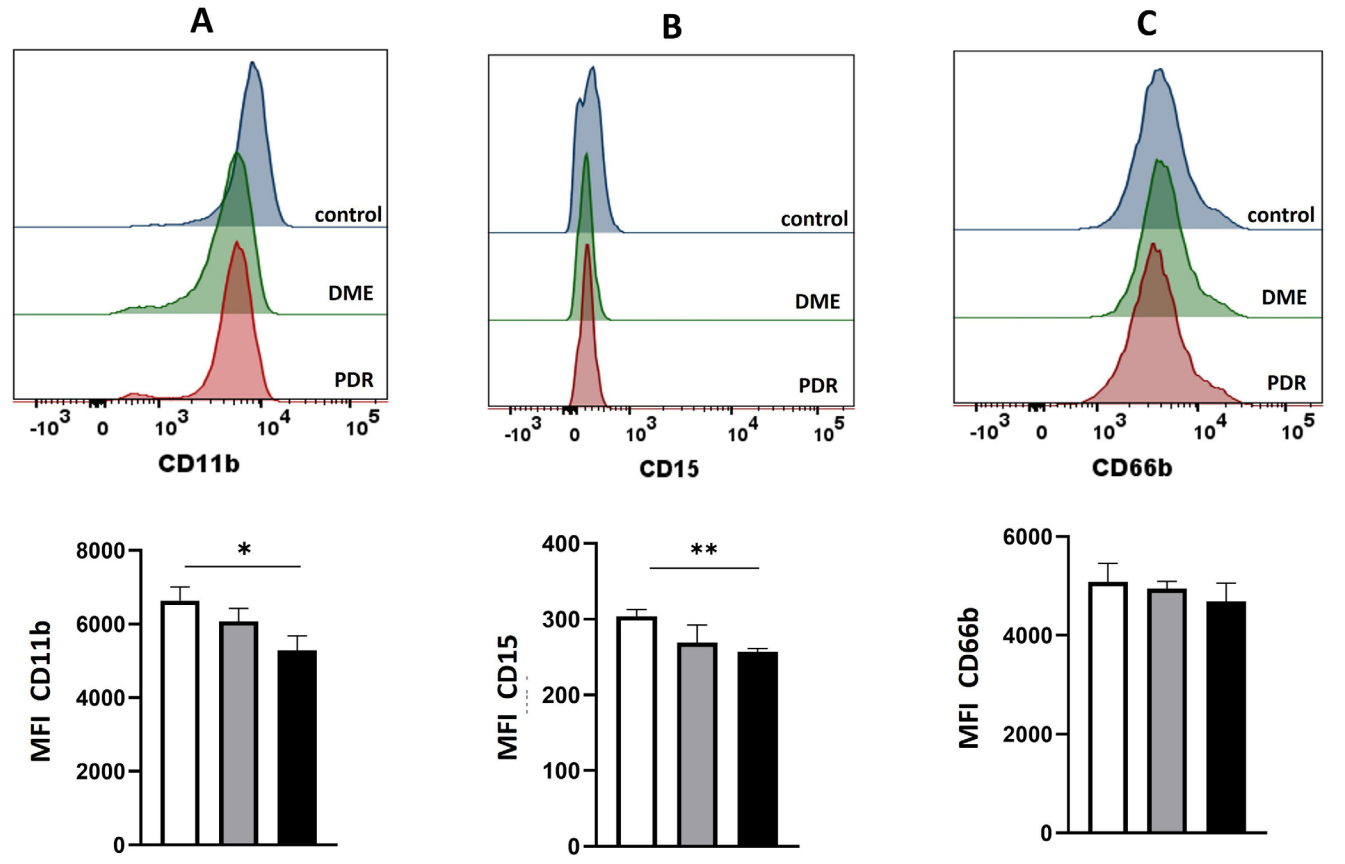

Figure 2. Vitreous samples obtained from patients with diabetic macular edema (DME) and proliferative diabetic retinopathy (PDR) modulate the activation markers of peripheral blood neutrophils. The upper panels show histograms of the expression of CD11b (**A**), CD15 (**B**), and CD66b (**C**) on neutrophils incubated with the vitreous of surrogate control (blue), DME (green), or PDR (red) patients. The lower panels are bar graphs of the medium fluorescence intensity (MFI) of the activation markers CD11b (**A**), CD15 (**B**), and CD66b (**C**) in peripheral neutrophils incubated with the vitreous of surrogate control (white bars), DME (gray bars), or PDR (black bars) patients. We found a significant decrease in both CD11b and CD15 activation markers in neutrophils exposed to the vitreous from patients with PDR. The bars represent the means ± standard error of the means. *$p < 0.05$. **$p < 0.01$.

neutrophils. It is secreted by various cell types, including epithelial, endothelial, and vascular smooth muscle cells; monocytes; macrophages; and adipocytes, in response to diverse stimuli, such as TNFα, IL-17, IL-1β, and lipopolysaccharide (LPS), among others [28]. In an experimental DM model, elevated CXCL5 levels were observed in the spinal cord, potentially linking CXCL5 levels to diabetic neuropathy [29] and underscoring its importance in this condition. Our data revealed a significant increase in CXCL5 levels (up to a sevenfold increase) in the vitreous fluid of patients with PDR, emphasizing the proinflammatory state associated with this condition. CXCL5 appears to be upregulated in DM, likely driven by inflammatory mediators such as TNFα [30], which also contributes to increased insulin resistance. Additionally, elevated urinary levels of CXCL5 have been reported in patients with DM who have nephropathy [31]. Notably, CXCL5 antibody treatment has been shown to improve insulin sensitivity [30]. These findings suggest that the increased CXCL5 levels in the vitreous fluid of patients with PDR further reinforce the inflammatory environment associated with this condition.

Angiogenin, also known as RNase 5, is an RNase initially identified as a vascularization molecule derived from adenocarcinoma cells [32]. It has an essential role in multiple biological processes, including stress adaptation, cell survival and communication, and stem cell homeostasis maintenance [33]. Serum angiogenin is considered a biomarker of cardiovascular diseases and plays a special role in oxidative stress, atheroma plaque instability, and inflammation [34]. Elevated plasma levels of angiogenin are associated with a greater risk of cardiovascular events in individuals with DM [35]. In our study, we observed elevated levels of angiogenin in the vitreous fluid of patients with PDR. This finding aligns with previous reports that demonstrated increased angiogenin levels in the vitreous of patients with DR [36], PDR, and proliferative vitreoretinopathy compared with idiopathic epiretinal membranes [37]. However, Marek et al. [38] reported low angiogenin levels in the vitreous of individuals with type 1 DM, which was consistent with reduced serum angiogenin levels. They suggested that angiogenin might not be directly involved in the pathogenesis of DR [38]. These discrepancies may be attributed to differences in DM types. Additionally, genetic polymorphisms among study populations could play a role and should not be excluded [39].

Interestingly, vitreous fluid from patients with DR was able to downregulate the expression of CD11b and CD15 neutrophil activation markers, whereas vitreous fluid from patients with DME did not significantly change the expression of these markers. Although NET release is closely associated with DR [18], neutrophil phagocytosis is impaired in DM and DR, increasing the susceptibility of individuals with these conditions to infectious diseases. Hyperglycemia enhances ROS production and autophagy; however, phagocytosis is decreased in peripheral neutrophils from individuals with DM [40]. These impairments are closely linked to alterations in the expression of activation molecules. Unexpectedly, in contrast to our previous findings [18], the current study revealed that CD11b and CD15 expression was downregulated in neutrophils incubated with vitreous from patients with DR and that no effect was observed on the DME vitreous. This finding contrasts with the well-documented elevation of CD11b and CD66b activation markers on monocytes and neutrophils in patients with type 2 diabetes mellitus, which promotes adhesion to the endothelium, exacerbates vascular damage, and contributes to systemic inflammation and injury [41]. We propose that this discrepancy may result from the diverse cytokine profiles present in the vitreous of patients with DR, potentially inducing an exhausted neutrophil phenotype. Similar phenomena have been observed in neutrophils from individuals with psoriatic arthritis [42] and cystic fibrosis [43], where in vitro neutrophil activation and function are significantly reduced. Although functional tests on neutrophils incubated with vitreous samples from PDR, DME, or control subjects—such as phagocytosis, NET release, or ROS determination—were not performed in this study, it is reasonable to assume that these functions might also be impaired in the DR context. To avoid nonspecific activation marker induction in neutrophils, we used whole peripheral blood, as neutrophil extraction methods induce neutrophil activation [44].

It is important to note that neutrophil activation is not exclusively driven by cytokine-mediated signaling but also involves other innate immune pathways, particularly the complement system. Activation products such as C3a and C5a are potent chemoattractants and activators of neutrophils, modulating their migration, degranulation, and oxidative responses through specific receptors. Although our study focused on the effect of the PDR and DME vitreous milieu on surface activation markers CD11b and CD15, we acknowledge that complement-mediated signaling may also contribute to neutrophil functional changes in the context of DR. Recent studies have highlighted the involvement of the complement system in the pathogenesis of DR and its potential to influence local immune cell activation [45]. Therefore, future investigations should include detailed profiling of complement components and receptor expression to better elucidate their role in neutrophil activation and potential exhaustion phenotypes observed in DR.

A limitation of this study is the absence of stratification of patients with PDR based on disease duration, the extent of retinal neovascularization, or prior panretinal photocoagulation at the time of vitreous sample collection. These clinical variables may influence the inflammatory composition of the vitreous and, consequently, neutrophil activation. Future studies should incorporate such stratification to better contextualize immune responses. Notably, none of the patients included in this study had received anti-VEGF therapy before vitreous sampling, thereby minimizing treatment-related variability in the immune profile.

Interestingly, the hematological inflammatory NLR, PLR, and MLR are used as predictive parameters associated with inflammatory diseases [46,47]. In the present study, we found high NLR and MLR in patients with PDR and DME compared with controls. Similarly, studies have shown that the levels of inflammatory cytokines, such as IL-6 and TNFα, are elevated in DR and associated with a higher NLR and PLR [48]; additionally, associations between MLR, NLR, and PLR and a worse prognosis of diabetic complications, such as diabetic foot ulcers [49] and peripheral arterial disease [50], exist. These findings suggest that inflammatory systemic and local processes may accelerate the development of DR, contributing to complications or worsening.

The findings of this work allow to understand the relevance of inflammation and its components, such as part of the physiopathology and progression of DR, and to identify inflammatory cytokines such as CCL2, CXCL5, and angiogenin as possible therapeutic targets together with vascular factors such as VEGF and platelet-derived growth factor (PDGF), which are commonly associated with degenerative vasculature growth in DR. Finally, this study provides novel insights into the functional modulation of peripheral neutrophils by the vitreous microenvironment in DR, a mechanism that remains underexplored. Our findings show that vitreous samples from patients with proliferative diabetic retinopathy PDR can downregulate key neutrophil activation markers, CD11b and CD15, on healthy donor neutrophils in an ex vivo setting. This suggests the presence of immunomodulatory or exhaustion-inducing factors within the diabetic vitreous milieu, which may contribute to dysregulated neutrophil function and inflammatory responses in DR.

Finally, this study provides novel insights into the functional modulation of peripheral neutrophils by the vitreous microenvironment in DR, a mechanism that remains underexplored. Our findings show that vitreous samples from patients with PDR can downregulate key neutrophil activation markers, CD11b and CD15, on healthy donor neutrophils in an ex vivo setting. This suggests the presence of immunomodulatory or exhaustion-inducing factors within the diabetic vitreous milieu, which may contribute to dysregulated neutrophil function and inflammatory responses in DR.

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