Perilipin 2–positive mononuclear phagocytes accumulate in the diabetic retina and promote PPARγ-dependent vasodegeneration

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Type 2 diabetes mellitus (T2DM), characterized by hyperglycemia and dyslipidemia, leads to nonproliferative diabetic retinopathy (NPDR). NPDR is associated with blood-retina barrier disruption, plasma exudates, microvascular degeneration, elevated inflammatory cytokine levels, and monocyte (Mo) infiltration. Whether and how the diabetes-associated changes in plasma lipid and carbohydrate levels modify Mo differentiation remains unknown. Here, we show that mononuclear phagocytes (MPs) in areas of vascular leakage in DR donor retinas expressed perilipin 2 (PLIN2), a marker of intracellular lipid load. Strong upregulation of PLIN2 was also observed when healthy donor Mos were treated with plasma from patients with T2DM or with palmitate concentrations typical of those found in T2DM plasma, but not under high-glucose conditions. PLIN2 expression correlated with the expression of other key genes involved in lipid metabolism (ACADVL, PLIN2) and the DR biomarkers ANGPTL4 and CXCL8. Mechanistically, we show that lipid-exposed MPs induced capillary degeneration in ex vivo explants that was inhibited by pharmaceutical inhibition of PPARγ signaling. Our study reveals a mechanism linking dyslipidemia-induced MP polarization to the increased inflammatory cytokine levels and microvascular degeneration that characterize NPDR. This study provides comprehensive insights into the glycemia-independent activation of Mos in T2DM and identifies MP PPARγ as a target for inhibition of lipid-activated MPs in DR.

Introduction

Type 2 diabetes mellitus (T2DM) affects a growing part of the population worldwide (1). Fifteen years after the onset of diabetes, more than 60% of patients will develop some form of diabetic retinopathy (DR) (2). DR stages are clinically characterized by the observation of retinal vascular damage (3). Alterations in the blood-retina barrier and the resulting progressive degeneration of retinal capillaries, ensuing retinal ischemia, extravasation, and bleeding occur in non-proliferative DR (NPDR), the early stage of the disease. Severe NPDR can ultimately progress to sight-threatening proliferative DR (PDR), which is characterized by the formation of neovascular tufts around the ischemic retinal tissue. These areas of vascular remodeling are associated with neurodegenerative processes and innate immune dysregulation (4, 5). High intraocular levels of inflammatory cytokines are found in patients with DR, and cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (CXCL8) have been proposed to be involved in the progression of DR (5, 6). Consistent with such a role for cytokines, treatments targeting inflammation have been shown to be protective against diabetes-induced neurodegeneration and vascular remodeling (7). Activation of retinal mononuclear phagocytes (MPs) has been described in patients with diabetes (8) and in animal models of T2DM (9, 10). The retinal MP population in DR is composed not only of resident microglia but also of infiltrating monocyte-derived (Mo-derived) macrophages. The latter play a prominent role in inflammation and vascular remodeling in DR (11–14).

DR is a multifactorial disease, and hyperglycemia only accounts for a limited proportion of the risk of developing DR (15). Indeed, a number of other glycemic-independent risk factors are associated with the development and progression of DR (16–18).
Dyslipidemia has been a largely neglected field of study but has recently attracted attention and is now considered to be an important area of research in DR (17, 18). Indeed, numerous studies (19–25), as well as a meta-analysis (26), have demonstrated correlations between plasma lipid levels and DR or diabetic edema in the past 10 years. Moreover, the lipid-lowering drug fenofibrate was shown to slow DR progression independently of glycemic control in 2 large randomized, controlled clinical trials (27, 28). Saturated free fatty acids (FFAs) are generally considered to be proinflammatory, whereas unsaturated FFAs are classically considered to be antiinflammatory (29–32). Palmitate (PA) (saturated 16-carbon FA [C16:0]) is the most abundant FFA in the plasma (33), and its concentration is found to be elevated among patients with T2DM (34, 35). MPs can take up and store lipids in intracellular lipid droplets stabilized by the amphiphilic protein perilipin 2 (PLIN2) (36). PLIN2+ lipid-laden MPs (PLIN2+ MPs) are found in atherosclerotic lesions and in fat lesions after trauma (37). In the brain, PLIN2+ MPs were found in grade 2 cerebellar infarcts, whereas they were absent in control and low-grade infarcts (37). More recently, lipid-laden MPs were found to progressively accumulate in the aging mouse and human brain (38). PLIN2+ MPs exhibit alterations in their metabolism, phagocytosis, and autophagy, leading to the production of ROS and inflammatory cytokines (38–42). The presence and role of MPs exposed to plasma lipids in the retina in DR remain to be determined.

In the present study, MPs expressing PLIN2 were found in the retinas of patients with DR in regions with active vascular leakage and vascular remodeling. Next, we aimed to understand the molecular cues that drive Mos to differentiate into PLIN2+ MPs and their role in vascular remodeling. We show that exposure of naive human Mos to heat-inactivated plasma from patients with diabetes or to FFAs was sufficient to induce PLIN2 expression and secretion of DR-associated inflammatory cytokines. Experimentally, we show that lipid-exposed MPs had strong vasodegenerative properties that were inhibited by a pharmacological PPARγ inhibitor. Overall, these results pave the way for potential treatment targeting MP PPARγ signaling in dyslipidemia-mediated inflammation, such as DR, and potentially beyond retinal vasodegeneration.

Results

Human diabetic retina postmortem analysis reveals PLIN2+ MP accumulation in regions with vascular leakage. PLIN2+ MPs have been shown to progressively accumulate in the brains of aging mice and humans and in regions of cerebellar infarcts. Lipid-laden macrophages have been reported in the retina following blood-retina barrier breakdown (43), but the localization of PLIN2+ MPs in the diabetic retina has not been reported thus far. We identified regions with potential PLIN2+ cells by staining retinas from a collection of postmortem diabetic donor retinas (Supplemental Table 1; supplemental material available online with this article; https://doi.org/10.1172/JCI161348DS1) with anti–collagen IV (COL4), anti-albumin (anti-ALB), anti-IBA1, a pan-MP antibody, and anti-PLIN2 antibodies. COL4 and ALB staining revealed regions where ALB was found only in the vessel lumen (Figure 1A) and regions with microaneurysms where extraluminal ALB was found (Figure 1B). IBA1 staining identified numerous MPs in contact with retinal neovascular cell, and 2 anti-PLIN2-stained samples showed iden-
levels by RNA-Seq. The expression level, in transcripts per million (TPM), of each transcript in each condition was determined (Supplemental Table 3), and the means are presented as scatter plots for the BSA condition (x axis) and the PA condition (y axis) (Figure 2G). DESeq2 analysis showed that PA treatment induced a statistically significant (adjusted P ≤ 0.05) differential expression of 9,794 transcripts (Supplemental Table 3). Among them, 528 (5.4 %) were regulated with a log_{2} fold change (FC) of greater than 2, of which 37 transcripts (0.4%, red dots) were upregulated more than 16-fold (Figure 2G). The top 10 up- and downregulated transcripts are shown in Figure 2H. We then performed a gene ontology (GO) enrichment analysis of these 528 regulated transcripts to determine the major functions affected by PA treatment. Thirteen GO terms were significantly enriched, 8 of which were related to lipid metabolism (red dots), consistent with the adaptation of Mo metabolism to the lipid-rich environment (Figure 2I). This observation was further confirmed by specific analysis of the broad GO term “fatty acid metabolic process” (414 genes). Among them, 229 of the 414 transcripts (55.3%) were found to be differentially expressed following PA versus BSA treatment. Sixteen (7.8%) were among the 37 highly regulated genes (FC ≥ 4) (Figure 2J and Supplemental Table 4). Only 2 were downregulated (SMPD3 and CD36; log_{2} FCs = –2.29 and –2.22, respectively) (Figure 2H and Supplemental Table 4). Our analysis confirmed the upregulation of PLIN2 and identified CXCXL8 as the third most highly expressed transcript in PA-stimulated Mos, with a log_{2} FC of 2.87 (Figure 2G, 2H, and J). PA stimulation also induced a strong upregulation of ANGPTL4 (log_{2} FC = 2.14). ANGPTL4 acts as an endogenous inhibitor of lipoprotein lipase (LPL), an enzyme responsible for breaking down triglycerides (TGs) in circulating lipoproteins, and its levels in aequous humor have been shown to correlate with areas of nonperfusion in patients with DR (45, 46).

Overall, our transcriptomic analysis showed a modification in lipid metabolism and DR-related cytokine expression in Mos in response to PA. We chose 4 markers that are highly regulated by PA exposure for downstream analysis: PLIN2 (for its role in intracellular lipid droplet stabilization, log_{2} FC = 4.13), PDK4 (an inhibitor of pyruvate conversion into acetyl-CoA, which was the most highly regulated transcript by PA treatment, log_{2} FC = 7.27), ACADVL (for its role in lipid β-oxidation, log_{2} FC = 2.7), and ANGPTL4 (for its role in regulating TG uptake and its involvement in DR physiopathology, log_{2} FC = 2.14) (Figure 2K). To ensure that our results were not biased by the ability of BSA to bind FFAs released by the cells, we also performed RNA-Seq analysis of gene expression in naive Mos stimulated with PA in the presence of BSA, compared with a control medium without BSA. PLIN2, PDK4, ACADVL, ANGPTL4, and CXCXL8 were also upregulated when MPs grown in BSA-free conditions were used as controls, and a very similar regulation of the genes involved in the “fatty acid metabolic process” was also observed (Supplemental Figure 1).

We then studied the effect on MP polarization of other FFA species that are also found to be elevated in patients with T2DM, such as stearate (SA) (C18:0), the second most abundant saturated FFA in the blood, and palmitoleate (PoA) (C16:1 n-7), an unsaturated form of PA. SA and PoA also demonstrated the ability to upregulate PLIN2, PDK4, ACADVL, ANGPTL4, and CXCXL8 transcripts (Supplemental Figure 2A) (34, 35). To gain a deeper understanding of the influence of FFA mixtures on MP polarization, we implemented an expanded set of stimulations. We then investigated the possible role of oleic acid (OA) (C18:1 n-9), one of the most abundant circulating FFAs traditionally considered to be antiinflammatory, in PA stimulation. Naive Mos were stimulated with an equimolar mixture of PA and OA (250 μM each, totaling 500 μM FFA), and transcript expression was compared with that induced by 500 μM PA alone. Interestingly, we found that the equimolar mixture significantly increased the expression of PLIN2 and PDK4 compared with expression following stimulation with PA alone, whereas, in sharp contrast, CXCXL8 was strongly downregulated. However, despite the presence of OA, we still observed overexpression of our selected markers PLIN2, PDK4, ACADVL, ANGPTL4, and CXCXL8 (Supplemental Figure 2B). Considering the complexity of the FFA milieu in vivo, we developed a custom FFA blend with a target concentration of 500 μM to allow a meaningful comparison with standard PA stimulation. According to Yi et al., the 4 most abundant FFAs in the blood are PA (136 μM), SA (46 μM), OA (117 μM), and linoleic acid (LA) (132 μM), collectively constituting 84% (430 μM) of the total FFAs (59 μM) (34). The custom blend of these 4 major FFAs was therefore formulated as follows to mimic their relative abundance in the bloodstream: PA, 157 μM; SA, 53 μM; OA, 136 μM; and LA, 153 μM (as detailed in Supplemental Table 5). As with the PA-OA equimolar mixture, this plasma representative blend also induced a trend toward higher expression of PLIN2, PDK4, and ACADVL (although these changes were statistically significant only for PDK4) when compared with 500 μM PA alone. Interestingly, ANGPTL4 was found to be significantly downregulated after FFA blend stimulation. Consistent with what we observed with the PA-OA equimolar mixture, the FA mixture also induced an overexpression of all of our selected markers (PLIN2, PDK4, ACADVL, ANGPTL4, and CXCXL8) compared with the BSA condition (Supplemental Figure 2B).

Last, we replaced PA with an unreadily metabolizable form of PA, PA_{1333}, in the blend. Under these conditions, we observed a nonstatistically significant trend toward lower expression of lip-
id-related transcripts, which correlated with a statistically significant reduction in expression of the inflammatory cytokine CXCL8 (Supplemental Figure 2B).

Stimulation of naive Mos with T2DM plasma increases the expression of PLIN2, which is correlated with the expression of lipid metabolism and DR marker genes. We next analyzed the expression of the key markers defined above in naive Mos exposed to plasma from patients with diabetes. We established a cohort of donors (Table 1 and Figure 3A) consisting of control non-diabetic (ND) donors (n = 10) and diabetic patients with no signs of retinopathy (T2DM no DR, n = 10), early-to-mild NPDR (T2DM NPDR, n = 10), or PDR (T2DM PDR, n = 7) and collected plasma from each (total: n = 10 ND donors and n = 27 patients with T2DM). The mean leukocyte count and plasma creatinine concentration were similar between the control and T2DM groups (Table 1). The patients with T2DM (n = 27) had been diagnosed with T2DM for a median duration of 15 years, and their median age was also higher than that of the ND donors (63 vs. 50 years) (Table 1). The median glycemia and glycated hemoglobin levels for the T2DM donors were 185 mg/dL and 8.4%, respectively, and 85 mg/dL and 4.85%, respectively, for the ND donors. The same purification of naive Mos from a healthy human donor was sampled and exposed respectively, for the ND donors. The same purification of naive Mos (Figure 3D). The expression of PLIN2, PDK4, ACADVL, and CXCL8 was again associated with the expression of lipid metabolism and DR marker genes.

In summary, our results show that heat-resistant components of T2DM plasma induce an expression pattern in human Mos very similar to that seen with PA. The lack of induction by glucose and the strong induction of lipid overload marker expression by the T2DM plasma strongly suggest that elevated plasma levels of FFA, such as PA, are responsible for the T2DM-induced shift in Mo polarization.

Table 1. Cohort structure

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No DM (n = 10)</th>
<th>T2DM (n = 27)</th>
<th>P value</th>
</tr>
</thead>
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<tr>
<td>Age (yr)</td>
<td>50 [34.75-50.50]</td>
<td>63 [55-66]</td>
<td>0.0002</td>
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<tr>
<td>Female sex (%)</td>
<td>80/20</td>
<td>56/44</td>
<td>0.0726</td>
</tr>
<tr>
<td>Male sex</td>
<td>20</td>
<td>44</td>
<td>0.0726</td>
</tr>
<tr>
<td>Glycemia (mg/dL)</td>
<td>85 [82-93]</td>
<td>185 [141-208]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>4.85 [4.28-5.20]</td>
<td>8.4 [7.40-11.30]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>T2DM duration (yr)</td>
<td>0 [0-0]</td>
<td>15 [10-20]</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BMI</td>
<td>25.85 [25.10-27.75]</td>
<td>27.30 [25.30-28.46]</td>
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<tr>
<td>TC (mg/dL)</td>
<td>207.0 [162.5-226.5]</td>
<td>191.5 [199.9-216.6]</td>
<td>0.8334</td>
</tr>
<tr>
<td>HDLc (mg/dL)</td>
<td>46 [41-50]</td>
<td>50 [37-68]</td>
<td>0.3935</td>
</tr>
<tr>
<td>VLDLc (mg/dL)</td>
<td>110.0 [92.5-140.0]</td>
<td>125.7 [87.6-144.9]</td>
<td>0.4726</td>
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<tr>
<td>Triglycerides (TG) (mg/dL)</td>
<td>143 [77.8-250.3]</td>
<td>175 [121-208.0]</td>
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<td>TC/HDLc (mg/dL)</td>
<td>4.3 [3.3-5.2]</td>
<td>3.60 [3.3-5.1]</td>
<td>0.5831</td>
</tr>
<tr>
<td>LDLc/HDLc (mg/dL)</td>
<td>2.5 [2.0-2.8]</td>
<td>2.4 [2.1-3.4]</td>
<td>0.7238</td>
</tr>
<tr>
<td>TG/HDLc (mg/dL)</td>
<td>2.8 [1.6-6.2]</td>
<td>3.5 [2.1-5.5]</td>
<td>0.8008</td>
</tr>
<tr>
<td>Leukocytes (per mL)</td>
<td>5935 [5262-6843]</td>
<td>6540 [6180-7680]</td>
<td>0.0857</td>
</tr>
<tr>
<td>Creatinine (mg/dL/serum)</td>
<td>0.70 [0.61-0.82]</td>
<td>0.86 [0.68-1.18]</td>
<td>0.0532</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>117 [108-125]</td>
<td>137 [130-146]</td>
<td>0.0012</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>72.5 [69.8-84.8]</td>
<td>81 [69-88]</td>
<td>0.4731</td>
</tr>
</tbody>
</table>

Results represent the median [25th-75th percentiles] for no DM (n = 10) and T2DM (n = 27; no DR = 10, NPDR = 10, PDR = 7) plasma donors. P values were determined using the Mann–Whitney U test (or Fisher’s exact test for the male/female ratio) between the no DM and T2DM groups. BP, blood pressure.
Dose-dependent and long-term effects of PA stimulation on naive Mo. We next explored naive Mo differentiation by assessing the effects of different concentrations and different durations of PA stimulation. Concentrations above 100 μM PA induced dose-dependent expression of PLIN2, PDK4, ACADVL, ANGPTL4, and CXCL8 transcripts after 42 hours (Figure 4, A and B). We next compared the expression of PLIN2 and CXCL8, which we demonstrated to be markers of lipid overload in early differentiating Mos, after 42 hours by Mos stimulated with PA for only the first 18 hours and compared the expression levels with expression by Mos continuously stimulated for 42 hours (Figure 4C). Medium renewal after 18 hours (PA-to-BSA condition) resulted in a reduction of PLIN2 expression compared with the PA-to-PA condition (PA exposure from t₀ to t₄₂ h), but CXCL8 expression remained elevated (Figure 4D).
Figure 4. Lipid-associated MPs produce inflammatory cytokines. (A) Schematic representation of naive Mo treatment with increasing concentrations of PA. (B) Nonlinear regression representation of the RT-qPCR relative expression of selected markers of healthy donor naive Mos treated for 42 hours with increasing concentrations of PA (0–500 μM). Values represent the mean ± SEM of 5 independent culture points. Goodness of fit is indicated by \( R^2 \) (0.9577, 0.9413, 0.7958, 0.9067, and 0.8298, respectively). (C) Schematic representation of 2-phase treatment of naive Mos to test the long-term effect of PA. (D) Scatter plot representation of RT-qPCR expression of PLIN2 and CXCL8 in healthy donor naive Mos subjected to the 2-phase treatment. Values represent the mean ± SEM of a minimum of 4 independent culture points. \( P \) values were determined by 1-way Welch’s ANOVA \( (P = 0.0010, 0.0002, 0.0001, 0.0010, 0.0002, 0.0001) \) followed by Dunnett’s T3 multiple-comparison test. (E) Schematic representation of the preparation CM of Mos. (F) Scatter plot of cytokines and growth factor protein concentrations in pg/mL detected by multiplex measurements in CM of naive Mos from a healthy donor differentiated with PA (PA-bound BSA, y axis) or BSA (unbound BSA, x axis) (left graph) or 25 mM glucose plus BSA (y axis) or 5 mM glucose plus BSA (x axis) (right graph).

Lipid-stimulated MPs secrete inflammatory cytokines. The presence of lipid-laden MPs in the vicinity of vascular leaks could be a source of vasoactive cytokines. We thus collected conditioned medium (CM) 42 hours after an initial 18-hour stimulation of naive Mos with PA or 25 mM glucose (Figure 4E). Using multiplex technology, the cytokine content of PA-stimulated PA-free CM (PAstimCMPAfree) (PA-to-BSA) was compared with CM from MPs previously stimulated with control CM (CtlCM) (BSA-to-BSA) or with the CM from MPs previously stimulated with 25 mM glucose (Figure 4E). Early PA stimulation upregulated the expression of the cytokines CCL2, FGF2, GMCSF, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IFN-γ, and TNF-α, which have been found to be elevated in the vitreous of patients with DR (5). By contrast, these cytokines were only slightly modulated or not expressed after a similar early stimulation with 25 mM glucose (Figure 4F).

These results highlight that key DR inflammatory cytokines were upregulated in the secretome of MPs after an initial stimulation with PA. We have previously showed that PAstimCMPAfree, the unreadily
Figure 5. Lipid-associated MPs show vasodegenerative properties. (A) Schematic representation of the preparation of CtlCM and PAstimCM<sub>PAfree</sub> from healthy donor Mos treated with PA (PA-bound BSA) or BSA (unbound BSA). (B and C) HUVECs were stimulated with either CtlCM or PAstimCM<sub>PAfree</sub>. (B) Epi-fluorescence images of HUVEC nuclei stained with Hoescht (white). Scale bars: 100 μm. (C) Scatter plot of normalized nuclei counts. Values represent the mean ± SEM of 6 independent culture points. The P value was determined using Welch’s 2-tailed t test. (D–G) Capillary degeneration was quantified in an ex vivo assay (48). (E) Time-course of the mean ± SEM sprout number between day 4 (D4) and day 8 (D8) in the 3 treatment groups: basal medium (n = 10, black), CtlCM (n = 7, blue), and PAstimCM<sub>PAfree</sub> (n = 7, red). (F) Violin plot of the log2 FC of sprout numbers between paired day-6 and day-8 rings; dots represent individual aortic rings, and dashed lines represent the median and quartiles. P values were determined by 1-way Welch’s ANOVA test (P < 0.0001) followed by Dunnett’s T3 multiple-comparison test. (G) Aortic rings and sprouts treated with CtlCM or PAstimCM<sub>PAfree</sub> on day 6 and stained with COL4 on day 8. Left: Epifluorescence micrographs of COL4 (white). Scale bars: 500 μm. Right: Higher-magnification confocal micrographs of COL4 (green). Scale bars: 200 μm. Nuclei were stained with Hoechst (blue). (H) Schematic of the biological changes in Mos after lipid exposure and their acquired properties.
metabolizable analog of PA, reduces lipid polarization of MPs (Figures 2E and Supplemental Figure 2B). We therefore hypothesized that PA_{CH3} could reduce the expression of a number of DR-related cytokines compared with PA. We thus examined the expression of CXCL8, TNF, IL1B, and IL6 and found that all of these inflammatory cytokine transcripts were significantly decreased when naive Mos were exposed to PA_{CH3} instead of PA (Supplemental Figure 4A).

Lipid-associated MPs show vasodegenerative properties. We tested the activity of the CM on human umbilical vein endothelial cells (HUVECs). PASTIMCM{PAfree} resulted in a 4-fold reduction in cell numbers after 24 hours compared with CtrlCM (Figure 5, A–C). We next quantified the capillary degeneration induced by PASTIMCM{PAfree} using our specially designed ex vivo assay (48). Rat aortic rings were allowed to sprout for 6 days and were then treated with PASTIMCM{PAfree}, Ctrl-CM, or basal medium. Sprouts from all rings were counted daily from day 4 (2 days before treatment) to day 8 (2 days after treatment) (Figure 5D). CtrlCM did not affect the sprouting rate of aortic rings. On the contrary, the addition of PASTIMCM{PAfree} resulted in a decrease in the mean number of branches starting from day 7 (Figure 5E), and a paired analysis of the number of branches between day 6 and day 8 showed severe loss of the organized endothelial cell network in each individual aortic ring (Figure 5F). Lectin-stained images of day-8 fixed aortic rings confirmed capillary degeneration after the addition of PASTIMCM{PAfree} (Figure 5G). Stimulation with PA_{CH3} did not correlate with increased expression of inflammatory cytokines (Supplemental Figure 4A). Conversely, PA_{CH3}STIM-CM{ASC17Tfree} (where naive Mos were initially stimulated with PA_{CH3} instead of PA) demonstrated significantly lower vasodegenerative activity compared with PAStimCM{PAfree} (Supplemental Figure 4, B and C). Given the design of our study, we concentrated only stable macromolecules larger than 10 kDa that were present in the CM. Consequently, our results suggest the likely involvement of macromolecules such as inflammatory cytokines secreted by MPs in the observed vasodegenerative properties (Figure 5H).

Inhibiting PPARγ signaling normalizes the PA-induced phenotype of differentiated MPs. We found a strong association between PLIN2 and CXCL8 expression in the presence of T2DM plasma or PA. However, we show that lipid removal led to a reduction in PLIN2 expression, whereas CXCL8 overexpression remained persistent, suggesting that the regulation of PLIN2 and CXCL8 may be uncoupled after the initial period of lipid exposure (Figure 4D). We therefore next aimed to determine whether an initial PLIN2 upregulation is necessary for CXCL8 induction. Given the short lifespan of primary human Mos and the challenges associated with their transfection with siRNA assays, we used as a model the more easily transfectable THP-1 cells, a human monocytic cell line derived from a patient with acute monocytic leukemia. The response of THP-1 cells transfected with a control siRNA to PA was similar to that of primary human Mos with a strong upregulation of PLIN2 and CXCL8 (Supplemental Figure 5, A and B). siRNA targeting PLIN2 reduced PLIN2 expression by 77% (Supplemental Figure 5A) and reduced PLIN2 induction in THP-1 cells by 69% in the presence of PA (Supplemental Figure 5B). This reduced expression of PLIN2 did not reduce inflammatory CXCL8 expression (Figure 6A) or lipid metabolism markers in THP-1 cells (Supplemental Figure 5C).

Therefore, we considered a broader approach targeting signaling pathways associated with lipid exposure. Our comprehensive bulk RNA-Seq analysis revealed that naive, healthy MPs swiftly responded to PA exposure by upregulating the expression of PPARγ, a gene encoding a member of the PPAR transcription factor family. Conversely, the expression of PPARα, which encodes the therapeutic target of fenofibrate—a clinical diabetes treatment—was marginally downregulated (log, FC = –0.44). Given the critical role of PPARs in controlling the transcription of lipid metabolism–associated genes, we hypothesized that a potentially large-scale, PPAR-mediated modulation of lipid-associated phenotypic characteristics occurs in MPs. Consequently, we examined the expression of PPARα and PPARγ transcriptional targets in PA-stimulated Mos. We assessed the expression of 448 high-confidence PPAR target genes that were identified by Fang et al. using a combination of transcriptomics data and the presence of the PPAR-responsive element (PPRE) in the regulatory regions of these target genes (49). We found that 252 of them (56.2%) were statistically differentially expressed after PA exposure (Figure 6B and Supplemental Table 6). Next, we analyzed the regulation of the 83 manually curated transcript targets of PPARα and the 104 manually curated transcript targets of PPARγ (49). PA regulated 38 transcripts (45.7%) on the curated PPARα target list, including 7 (8.4%) with a log, FC of 2 or higher (Figure 6B and Supplemental Table 7). PA also regulated 50 transcripts (49.0%) on the curated PPARγ list, including 6 (5.8%) with a log, FC of 2 or higher (Figure 6B and Supplemental Table 8). Although ACADVL was not included in the curated PPAR target list, the other 3 previously identified PA-associated transcripts (PLIN2, PDK4, and ANGPTL4) were all represented in the highly regulated target list (Figures 2 and 3). These findings suggest that PPARα and PPARγ are potential targets to blunt lipid-induced proinflammatory MP polarization.

We therefore tested the effects of fenofibric acid and GW6471 (a PPARα agonist and antagonist, respectively) and pioglitazone and T0070907 (a PPARγ agonist and antagonist, respectively) on the expression of the selected prototypical CXCL8 transcript (Figure 6, C–G). Neither the PPARα agonist nor the antagonist affected CXCL8 expression in the BSA condition, and these agents failed to reduce its induction after PA stimulation (Figure 6, D and E). Pioglitazone, a PPARγ agonist, increased CXCL8 expression in the BSA condition and had no effect after PA stimulation (Figure 6F). Finally, the PPARγ antagonist T0070907 strongly reduced CXCL8 expression after PA stimulation and also reduced its basal expression in the BSA condition (Figure 6G). Consistent with these results, T0070907 reduced the expression of the PPAR targets PLIN2, PDK4, and ANGPTL4 but not of the non-PPAR–curated target ACADVL (Figure 6H). CXCL8 downregulation was associated with decreased expression of the DR-associated cytokines TNF, IL1B, and IL6 (Supplemental Figure 6).

We have previously shown that PAStimCM{PAfree} leads to a reduction in the number of HUVECs relative to CtrlCM (Figure 5, A–C). To gain further knowledge about the potential protective effect of T0070907 on endothelial cell viability, we next analyzed HUVEC apoptotic cell death by TUNEL assay 48 hours after addition of CtrlCM, PAStimCM{PAfree}, or (PA+T0070907) stimCM{PA+T0070907free}. PASTIMCM{PAfree} induced a statistically significant 75% increase in TUNEL+ HUVECs compared with CtrlCM,
from patients with T2DM (with or without DR) strongly upregulated the expression of lipid overload markers in Mos from healthy donors, which strongly correlated with the expression of DR biomarkers such as \textit{ANGPTL4} and \textit{CXCL8}. We also show that PA, an abundant plasma FFA that is elevated in dyslipidemic plasma of patients with T2DM, induced a nearly identical expression pattern in naive human Mos. We characterized the PA-induced transcriptome and secretome changes and show that the PA-stimulated Mo secretome induced endothelial apoptosis and ex vivo capillary degeneration. A PPAR\textsubscript{γ} antagonist blunted PA-induced cytokine expression and reversed the vasodegenerative properties of the PA-stimulated Mo secretome.

Discussion

In this study, we show that PLIN2\textsuperscript+ MPs were present in the vicinity of leaky capillaries, identified by extraluminal ALB, in postmortem human T2DM donor retina with histopathologic features of NPDR. Importantly, we demonstrate that hi-plasma whereas (PA+T0070907)stimCM\textsubscript{PA+T0070907}\textsubscript{free} completely rescued HUVECs undergoing apoptotic cell death (Figure 6I). Finally, we compared the effect of these 3 CMs on the integrity of the capillary network in the rat aortic ring model. Again, we found that (PA+T0070907)stimCM\textsubscript{PA+T0070907}\textsubscript{free} was protective of the vascular network compared with PAstimCM (Figure 6J).
Plasma lipids are internalized by Mos. FFAs are abundant in the blood, and their levels are elevated in T2DM plasma (34, 50). TGs, another lipid component found in the blood, are composed of 3 FAs that can be hydrolyzed by extracellular LPL and further increase the pool of FFAs available to cells. Lipoprotein TGs and ALB-bound FFAs do not extravasate in the absence of vascular leakage. However, years of diabetes can compromise the integrity of the blood-retina barrier, leading to increased permeation of plasma components such as ALB, carbohydrates, and lipids into the retinal environment. These elements not only come into direct physical contact with local cells such as Müller glial cells, leading to their robust activation (51, 52), but also shape the milieu that drives the differentiation of infiltrating Mos. Using chromatographic techniques, we demonstrated that Mos internalized PA. We show that the proportion of PA chains increased markedly, while the proportion of other FA chains did not change. Together with the increased expression of PLIN2, this suggests that PA was stored intracellularly. Interestingly, we show that Mo activation persisted even after PA removal. This long-term effect suggests that the resolution of in vivo inflammation may lag even after extravasated lipids have been taken up intracellularly.

Clinically, elevated plasma TG levels have been associated with an increased risk of DR, with a clear association between TG levels and an increased frequency of hard exudates (53). We performed classical lipid analysis of plasma from our donors in the clinic (total cholesterol [TC], HDL cholesterol [HDLc], LDLc, VLDLC, and TGs) and observed no significant difference in overall plasma lipid load between the controls and T2DM donors in our cohort. However, we found that plasma-derived nonglucose cues could induce lipid-associated MP polarization. Our study thus suggests that there may be undiagnosed risks (i.e., the available PA that we showed had a detrimental effect on Mo polarization) of Mo activation that cannot be detected by performing classical lipid analysis in patients with T2DM. Our study focused on PA, the most abundant FFA in the blood. PA is not the only elevated FFA in diabetes, and numerous FFAs are also found to be elevated in T2DM (34, 35). We also show that SA, the second most abundant saturated FFA in the blood, PoA, the n-7 unsaturated form of PA, and a plasma representative lipid blend also induced PLIN2 overexpression.

Lipid-laden MPs are associated with vascular remodeling during DR. PLIN2+ MPs are found in atherosclerosis plaques, cholesterol polyps of the gall bladder, and fat necrosis (37). In the present study, in DR, we found PLIN2+ MPs in regions of microvascular disease characterized by ALB extravasation, suggesting that MPs were involved in the uptake of extravasated lipids and lipid storage. A similar observation has been made in the central nervous system, where PLIN2+ cells accumulate with age in the hippocampus and can be stained with lipid dyes (38). Consistent with our observation, MPs have been found in the red oil1 hard exudates of the retina in DR (54). More recently, adaptive optics funduscopy has revealed the presence of round motile particles that resemble MPs in hard exudates, a structure found in the periphery of regions of active vascular leakage (43). Collectively, these findings indicate that lipid-polarized MPs are found in regions of active vascular remodeling in the DR retina, that they may progressively accumulate in regions exposed to plasma leakage, and that they could be a source of vasoactive cytokines.

In an effort to understand how plasma leakage may influence MP activation, in particular the fate of infiltrating Mos, we differentiated naïve Mos in the presence of hi-plasma from healthy and T2DM donors. We show that hi-plasma from patients with T2DM rapidly induced a lipid-associated polarization of MPs characterized by overexpression of lipid-related genes. We also show a correlation between PLIN2 expression (used as a proxy for MP lipid load) and other lipid-related genes as well as DR biomarkers such as ANGPTL4 and CXCL8.

Such differentiation of Mos could be mimicked by PA, but not by glucose exposure. PA stimulation was associated with an increase in the production of at least 14 proinflammatory cytokines, 11 of which have been shown to be directly involved in DR. Similarly, Müller glial cells, the major glial cells of the retina, have been shown to have a greater increase in the expression of cytokines associated with DR when stimulated with PA than with glucose (51, 52).

ANGPTL4 is a protein that regulates lipid processing but also has vasoactive properties. ANGPTL4 was notably upregulated when naïve Mos were stimulated with PA or T2DM plasma, and ANGPTL4 expression showed a strong correlation with PLIN2 expression. ANGPTL4 plays an important role in the pathogenesis of DR (55, 56). Its expression has been found to correlate with the nonperfused area (45); in line with this finding, we found that the net effect of the PA-stimulated MP secretome was vasodegenerative, which was highlighted by a decrease in vascular sprouting in a 3D model and an increase in endothelial cell apoptosis.

Interestingly, we found that MPs remained activated and secreted inflammatory cytokines for at least 24 hours after the initial lipid stimulation. This effect may lead to a long-term vascular remodeling activity of lipid-stimulated MPs. Such vasodegenerative activity is consistent with the results of previous reports showing an overall antiangiogenic activity of MPs in the context of retinal ischemia. Indeed, MPs are associated with early vascular regression in models of ischemic retinopathy and contribute to the resolution of vascular proliferation (10, 57). In our hands, mouse Mos were less sensitive to PA than were human Mos, and our attempts to establish a mouse model of lipid-associated DR have so far been unsuccessful. This difference is consistent with the overall difference in the severity of DR between mice and humans. Mice do not develop clear early signs of NPDR, such as severe retinal capillary loss or massive blood retina-barrier breakdown and thus are not prone to progress to PDR like humans are. Hence, although our ex vivo aortic ring model clearly identified vasodegenerative properties of lipid-exposed MPs, this could not yet be validated in vivo. In contrast, subretinal MPs in exudative age-related macular degeneration show proangiogenic activity (58, 59). The exact mechanism by which the addition of CM from PA-stimulated MPs leads to endothelial cell loss is yet to be determined. Cytokines found to be increased in CM from PA-stimulated MPs (CCL2, FGF2, IL-1β, IL-2, IL-4, IL-5, IL-6, CXCL8, IL-10, IFN-γ, and TNF-α) have been implicated in the pathogenesis of DR and are probably responsible for this activity.
The PPARγ signaling pathway is involved in vasodegenerative activities of lipid-associated MPs. We demonstrated a potent correlation between the expression of PLIN2 and CXCL8 during the early stages of MP differentiation in the context of plasma lipids and exposure to various isolated FFAs. This may suggest a regulatory role for PLIN2 in CXCL8 expression. Similar correlations between PLIN2 and inflammatory cytokines have been observed in tumor-associated macrophages as well as in THP-1 cells (39, 60). Notably, PLIN2+ MPs are observed around retinal microaneurysms and in numerous pathological contexts, suggesting that therapeutic inhibition of PLIN2 may serve as a strategy to curb inflammation-associated vascular remodeling. However, our results show that siRNA-based silencing of PLIN2 transcript expression in the THP-1 cell line, a model of Mos, neither inhibited CXCL8 transcription nor altered the regulation of other selected markers of lipid metabolism. Similarly, the production of cytokines by bone marrow-derived macrophages stimulated with various lipids remained unaltered following PLIN2 deletion (61), whereas Chen et al. reported a slight reduction in inflammatory cytokine expression in unstimulated THP-1 cells following PLIN2 silencing (39). Altogether, these results suggest that, while PLIN2 serves as an excellent marker of lipid polarization in vivo in the human retina and in vitro with Mos stimulated by T2DM plasma or PA, its inhibition by siRNAs is insufficient to prevent inflammatory polarization of at least lipid-exposed THP-1. Considering that we observed persistent overexpression of CXCL8, even after PA removal, and given the high stability of PLIN proteins when incorporated into lipid droplets (62, 63), therapeutic strategies targeting the inhibition of PLIN2 protein production may prove ineffective in situations in which MPs harboring lipid droplets have already accumulated.

Our transcriptomics analysis of differentiating Mos in the presence of PA or plasma from patients with T2DM showed upregulated expression of the genes PLIN2, PDK4, and ANGPTL4. These genes are all transcriptional targets of PPAR transcription factors. Consistent with this role, our RNA-Seq analysis identified PPARG as one of the most highly upregulated genes (log, FC = 4.07), whereas PPARα was slightly downregulated (log, FC = −0.44). Members of the PPAR family are nuclear transcription factors that bind to the retinoid X receptor (RXRα) to regulate the expression of numerous genes, most of which are part of the lipid metabolism pathway. PPARs act as repressors when not bound to a ligand. When natural (i.e., FFA agonists) or synthetic ligands bind to the ligand-binding domain (LBD) of PPARs, they promote the binding of coactivator or corepressor factors, resulting in ligand-dependent modulation of gene expression. We investigated the role of the PPARs and PPARγ pathways in the differentiation of Mos in the presence of lipids using PPARα and PPARγ agonists and antagonists. Fenofibrate, which is metabolized to the PPARγ agonist fenofibric acid, is a widely used systematic treatment to lower plasma lipid concentrations. Fenofibrate has successfully proven its protective effect in patients with DR (27, 28). It has also been shown to reduce systemic inflammation in patients with NPDd, suggesting a potential role of circulating lipids or lipid metabolism in the chronic inflammatory status of patients with T2DM (64). Fenofibric acid and GW6471, a PPARα antagonist, did not regulate the expression of the DR prototypical cytokine CXCL8 in MPs. However, in animal models of T1DM, systemic fenofibrate treatment has a protective effect on the blood-retina barrier and protects against neovascularization in the oxygen-induced retinopathy model, notably by attenuating the overexpression of VEGF, CCL2, and ICAM-1 (39). Overall, this suggests that the beneficial effects of PPARα agonists in vivo could be due to effects on nonmyeloid cells. For example, PPARα agonists have been shown to directly protect endothelial cells (65, 66).

In contrast to the PPARα agonist T0070907, a PPARγ antagonist, attenuated PA-induced CXCL8 overexpression and the CM of naive Mos treated with PA+T0070907 showed reduced Vasodegenerative properties. Activation of the PPARγ pathway in MPs is generally considered to be antiinflammatory (67, 68), and there is consensus for a beneficial effect of systemic PPARγ agonist treatment in a wide range of diseases, including diabetes and its comorbidities. However, PPARγ antagonism has been reported to protect against the progression of nonalcoholic fatty liver disease (NAFLD) (69) and to reduce foam cell formation (70–72). In BV2 microglia cells treated with LPS, and thus in the absence of DR-relevant stress, Pparγ knockdown or T0070907 treatment was also shown to be associated with antiinflammatory polarization (73). In a study using the human myeloid cell line Mono Mac 6, the small molecule bindarit was shown to promote, in a PPARγ-dependent manner, overexpression of the lipid-associated protein FABP4 along with the CXCL8 transcript (74). In accordance with this result, we found that pioglitazone, a PPARγ agonist, increased the expression of CXCL8 in the BSA-only condition, suggesting that PPARγ upregulation may result in adverse effects even in the absence of exogenously added PPAR ligand. Locally in the eye, inflammation in retinal pigment epithelial cells treated with N-retinylidene-N-retinylethanolamine, a byproduct of the visual cycle associated with age-related macular degeneration, was also shown to be inhibited by T0070907 (75). A possible explanation for this discrepancy between the beneficial effects of PPARγ agonists and antagonists may be that normalization of circulating lipid levels and restoration of lipid storage in adipocytes by systemic PPARγ agonism is responsible for the observed beneficial effect, whereas in our setting, PPARγ antagonism was applied only to MPs. Overall, growing evidence suggests that the definition of PPARγ pathway activation as an inhibitor of M1 inflammatory polarization and an inducer of M2 antiinflammatory polarization may be an oversimplification. The mechanism by which these plasma lipids induce Mo differentiation into MPs with proinflammatory properties is still unknown. However, and in accordance with our finding that PA was rapidly internalized, PA-induced polarization of MPs to a proinflammatory profile has recently been shown to be independent of direct TLR4 binding (76). Our results further suggest that the differentiation of Mos in the presence of lipids leads to a modification of their metabolism, as (a) the use of BAP3, a PA analog that can be internalized but that is not readily metabolizable, did not trigger CXCL8 secretion, nor did it induce the degeneration of aortic ring sprouts, and (b) the PDK4 gene was found to be highly upregulated in the presence of PA, suggesting a potential switch to aerobic glycolysis (the so-called Warburg effect), a situation found in proinflammatory MPs. Of note, PPARδ antagonism is also beneficial in the ocular context. The lipid-induced Müller glial cell response can be inhibited by GSK0660, a PPARδ antag-
onist (77). Overall, this suggests that PPAR antagonists could be used locally in addition to systemic PPARα agonist treatment to inhibit the pathological differentiation of local and infiltrating cells in the retina in DR.

**Study limitations.** In this study, we focused on identifying the major class of lipids present in the blood of our cohort of patients. However, we must acknowledge that detailed information regarding lipid chain composition and concentrations of specific FFAs, including PA, was not obtained. Future investigations will be necessary to establish possible correlations between individual plasma lipid species, the activation level of naive Mos, and DR and its progression.

Additionally, our findings demonstrated that the secretome of PA-stimulated MP exhibits antiangiogenic activity, suggesting a potential link between lipid extravasation and vascular degeneration in vivo. However, it is essential to recognize that our study lacked relevant in vivo models of vascular permeability. As a result, we were unable to assess the relative importance of MP-induced degeneration compared with other known circulating factors involved in vascular remodeling, such as high glucose, advanced glycation endproducts (AGEs), oxidized lipids, and metabolites. More recently, diabetes-induced microbiome modification has also been recognized as an important player in disease progression. Exploring additional in vivo models and investigating the interactions of various factors contributing to vascular remodeling will provide valuable insights into this complex phenomenon.

In conclusion, DR is a neurodegenerative, microvascular, and inflammatory complication of T2DM, in which breakdown of the blood-retina barrier and vascular degeneration lead to the extravasation of plasma proteins, lipids, and leukocytes. We showed that MPs expressing PLIN2 were found in retinas with active vascular leakage and vascular remodeling. Exposure of naive Mos to hi-plasma from patients with diabetes or PA was sufficient to induce PLIN2 expression and trigger long-term expression of DR-related proinflammatory cytokines. The secretome of lipid-exposed naive Mos showed strong vasodegenerative properties that could be blunted by a PPARγ inhibitor. Our study sheds light on the harmful role of lipid-associated MP accumulation in the retina in DR and the role of pathogenic lipids, rather than glucose, in the activation of these cells.

**Methods**

Detailed Methods can be found in the Supplemental Methods.

**FFA solubilization and medium preparation.** To allow FFA solubilization in culture medium, FFAs were bound to FFA-free BSA. FFAs were dissolved in absolute ethanol (EtOH) and added to a BSA-containing culture medium (0.88% w/v of BSA) to obtain the working concentration (500 µM FFA, 0.5% v/v EtOH; FFA/BSA molar ratio of 3.8).

**Preparation of CM.** To obtain CM free of BSA and PA, Mos were isolated as described in the Supplemental Methods and stimulated for the first 18 hours. The culture medium was then removed, and early differentiated MPs were cultured for another 24 hours in a fresh control culture medium.

**Data and materials availability.** Experimental materials are available upon request with no restrictions. The RNA-Seq raw fastq files and count files were deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE239512). Values for all data points in graphs can be found in the Supplemental Supporting Data Values file.

**Author contributions**

GB, RK, FB, and XG conceived the study. GB, HCM, FB, AJC, and XG curated data. GB, HCM, FS, and XG acquired funding. GB, RK, LP, TMS, HCM, PN, SA, FPMR, LV, and XG collected data. GB, RK, and XG designed the study methodology. AC, DRDLP, AJC, YG (patient recruitment and phenotyping), NA, (lipidomics), and JAS provided resources. GB, NA, YG, FS, and CD, and XG supervised the study. GB and XG wrote the original draft of the manuscript. GB, RK, HCM, RK, FPMR, DRDLP, AJC, CD, YG, FS, and XG reviewed and edited the manuscript.

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**Statistics.** GraphPad Prism 8 (GraphPad Software) was used for all graphical representations and for all but the RNA-Seq statistical analysis. A detailed explanation of the specific statistical choices made is provided in the Supplemental Methods. A P value of less than 0.05 was considered significant.


