Patrolling Monocytes Are Recruited and Activated by Diabetes to Protect Retinal Microvessels

Francesco Tecilazich,[1,2,3] Toan A. Phan,[1] Fabio Simeoni,[4] Giulia Maria Scotti,[4] Zeina Dagher,[1,2] and Mara Lorenzi[1,2]

Diabetes 2020;69:2709–2719 | https://doi.org/10.2337/db19-1043

In diabetes there is a long latency between the onset of hyperglycemia and the appearance of structural microangiopathy. Because Ly6C^{low} patrolling monocytes (PMo) behave as housekeepers of the vasculature, we tested whether PMo protect microvessels against diabetes. We found that in wild-type mice, diabetes reduced PMo in the general circulation but increased by fourfold the absolute number of PMo adherent to retinal vessels (leukostasis). Conversely, in diabetic NR4A1$^{-/-}$ mice, a model of absence of PMo, there was no increase in leukostasis, and at 6 months of diabetes, the number of retinal acellular capillaries almost doubled compared with diabetic wild-type mice. Circulating PMo showed gene expression changes indicative of enhanced migratory, vasculoprotective, and housekeeping activities, as well as profound suppression of genes related to inflammation and apoptosis. Promigratory CXCR4 was no longer upregulated at longer duration when retinal acellular capillaries begin to increase. Thus, after a short diabetes duration, PMo are the cells preferentially recruited to the retinal vessels and protect vessels from diabetic damage. These observations support the need for reinterpretation of the functional meaning of leukostasis in diabetes and document within the natural history of diabetic retinopathy processes of protection and repair that can provide novel paradigms for prevention.

Diabetes induces functional and structural changes in microvessels that lead to a characteristic microangiopathy, which, in turn, underlies the chronic complications of diabetes. However, there is a long latency period between the onset of hyperglycemia and the appearance of diabetic retinopathy, usually the first microvascular complication to become manifest (1,2). This latency does not imply, but is consistent with, the presence of mechanisms for vascular protection and repair.

One of the first observable effects of experimental diabetes in retinal vessels is leukostasis, consisting in an increased number of white blood cells (WBC) firmly adherent to the vascular endothelium. Leukostasis is detectable before any vascular pathology (3) and is traditionally interpreted as an inflammatory event causally related to the development of diabetic retinopathy (4). In diabetic rodents, monocytes are prominent contributors to leukostasis in retinal vessels (5).

Monocytes are circulating mononuclear leukocytes that can be divided in two principal subsets based on their functional and morphological characteristics: the classical inflammatory monocytes (CCR2^{high}Ly6C^{+} in mice; CCR2^{high}CD14^{+} CD16^{-} in humans), and the nonclassical patrolling monocytes (PMo) (CX3CR1^{high}Ly6C^{-} in mice; CX3CR1^{high}CD14^{dim}CD16^{+} in humans) (6). PMo survey normal blood vessels by crawling along the endothelium (7), produce noninflammatory or anti-inflammatory cytokines (8,9), and do not produce reactive oxygen species (8). PMo exert housekeeping functions for the endothelium and have been named "accessory cells" of the endothelium (10). While it is known that diabetes activates the innate and adaptive immune systems (11), little is known about its effects on PMo. We entertained the hypothesis that diabetes activates PMo and that their functional characteristics would lead to beneficial consequences for the retinal vessels.

[1]Schepens Eye Research Institute of Massachusetts Eye and Ear, Boston, MA
[2]Department of Ophthalmology, Harvard Medical School, Boston, MA
[3]Istituto di Ricovero e Cura a Carattere Scientifico Burlo Garofalo, University of Trieste, Trieste, Italy
[4]Center for Translational Genomics and Bioinformatics, Istituto di Ricovero e Cura a Carattere Scientifico San Raffaele, Milan, Italy
Corresponding author: Francesco Tecilazich, francescotecilazich@gmail.com

Received 15 October 2019 and accepted 28 August 2020

This article contains supplementary material online at https://doi.org/10.2337/figshare.12896681.

Here we show that in diabetes, PMo are recruited to the retinal microcirculation and activate a protective biosynthetic program. In addition, diabetic mice lacking PMo developed more severe retinal microangiopathy than diabetic wild-type (WT) controls. These findings point to a vasculoprotective role of PMo in the context of leukostasis.

RESEARCH DESIGN AND METHODS

Animals

Heterozygous *Nr4a1*$^{+/-}$ mice on a C57Bl/6J background purchased from The Jackson Laboratory were bred to generate *Nr4a1*$^{-/-}$ mice and *Nr4a1*$^{+/+}$ WT littermate controls. The NR4A1$^{-/-}$ mouse is an animal model of absence of PMo (12). Male mice were randomly assigned to a diabetic or control group. Diabetes was induced at 6–8 weeks of age with the standard protocol of multiple streptozotocin injections (50 mg/kg body wt) administered intraperitoneally on 5 consecutive days. Diabetes was diagnosed if blood glucose was ≥250 mg/dL. Diabetic mice were weighed every other day to determine the need for administration of insulin (0.2–0.4 units NPH, s.c.) and 0.9% saline (1–2 mL, s.c.) to prevent catabolism and dehydration, respectively, while maintaining hyperglycemia. All animal experiments were conducted in accordance with the Schepens Institutional Animal Care and Use Committee guidelines (protocol No. S-372-0616).

Flow Cytometry and Sorting Experiments

Peripheral blood was collected between 8:00 A.M. and 10:00 A.M. by submandibular bleeding into EDTA tubes and processed as described in the Supplementary Material. PMo were identified as CD45^{hi}/CD11b^{hi}/Lin(CD3/CD19.9/NK1.1)$^{-}$/Ly-6G^{-}/CD115^{hi}/Ly-6C^{-} and sorted with a FACSAria II (BD Biosciences, Franklin Lakes, NJ) (Supplementary Fig. 1).

Intracardiac Perfusion and Quantitation of Leukostasis

The experiments followed published procedures (13) with the addition of a system of checkpoints for quality control (see Supplementary Material). Mice were deeply anesthetized with an intraperitoneal injection of a ketamine (120 mg/kg) and xylazine (10 mg/kg) solution and positioned under the microscope on a heating pad to maintain physiological body temperature. A large incision was made into the right atrium, and the perfusion fluids maintained at 37°C were delivered via a peristaltic pump through a 26-gauge needle into the left ventricle: 10 mL PBS to wash away blood (prewash); 10 mL PBS containing concanavalin A (1 mg) conjugated to fluorescein (FITC-ConA) to label luminal endothelium and the cells remaining adherent, and 10 mL PBS to wash away the unbound FITC-ConA (postwash). The eyes were enucleated, rinsed in PBS, and immediately fixed in 4% paraformaldehyde for 1 h. The retinas were dissected and whole-mounted on slides. The FITC-ConA^{+} cells were counted on the retinal whole-mounts throughout the three capillary layers under a fluorescence microscope. In some experiments, one of the two retinas was further processed for immunohistochemistry.

Immunohistochemistry

Fixed whole retinas were placed in blocking buffer (10% donkey serum for anti-CD45 or 10% goat serum for anti-CD16.2, 1% BSA, and 1% Triton X-100 in 1× PBS) for 2 h at room temperature. The whole retinas were then incubated with the primary antibody rat anti-mouse CD45 or Armenian hamster anti-mouse CD16.2 in blocking buffer at 4°C overnight. After washes, incubation with secondary antibodies, and further washes, the retinas were mounted vitreous side up on a glass slide. Details of antibodies and procedures are reported in Supplementary Material.

Fluorescein Angiography

Among the different methods to measure retinal vascular permeability in diabetic rodents (14), fluorescein angiography (FA) and MRI are the only ones applicable to live animals. We used FA to be able to measure retinal vascular leakage in the same mice to be used for counts of acellular capillaries. Procedures are described in Supplementary Material.

Acellular Capillaries

To assess degeneration of retinal vessels, we counted acellular capillaries. Retinal trypsin digests were prepared using procedures previously described in detail (15) and summarized in the Supplementary Material.

RNA Purification, RNA Sequencing, and Differential Expression Analysis

RNA was isolated using Trizol-LS from ∼20,000 PMo sorted by FACS from the circulation of each of three diabetic and three control WT mice. cDNA was synthesized and RNA sequencing (RNA-Seq) libraries were constructed using the SMARTer v.3 kit (Clontech Laboratories, Mountain View, CA). Sequencing was performed on Illumina HiSeq 2500, resulting in ∼30 million of paired-end 50 base-pair reads per sample. Reads were aligned to the reference transcriptome (mm9 assembly) using STAR aligner. Read counts were calculated using HTSeq v.0.6.0 based on ensemble annotation for National Center for Biotechnology Information 37/mm9 assembly. Differential expression analysis was performed using EdgeR package, classifying as differentially expressed genes (DEGs) those genes that changed twofold or more and had a false discovery rate (FDR) of ≤0.05.

Enrichment Analysis

Enrichment analysis was performed on DEGs using the EnrichR web platform (https://amp.pharm.mssm.edu/Enrichr) for the preliminary selection of pathways. Selected gene sets were further analyzed using preranked Gene Set Enrichment Analysis (GSEA) (16) on all the expressed genes. Genes were preranked using the log2 fold change of the expression values between diabetic and control PMo. Gene sets included in the GSEA were obtained from the Molecular Signatures Database.

Quantitative PCR

Quantitative (q)PCR was performed on cDNA from circulating PMo sorted by FACS from diabetic and nondiabetic

WT mice, using the ViiA 7 real-time PCR system (Thermo Fisher Scientific) and a TaqMan Gene Expression Master-Mix and TaqMan primers. Data were analyzed on the basis of the relative expression method.

Statistical Analysis

The specific tests used to analyze each set of experimental results are indicated in the figure and table legends. Normally distributed continuous data were analyzed using a two-tailed Student *t* test to compare two groups and one-way ANOVA to compare several groups, followed by the Bonferroni multiple comparison test. All statistical analyses were performed using Stata 12 software (StataCorp LLC, College Station, TX). Scatterplots were designed using Stata 12 or GraphPad Prism (GraphPad Software, San Diego, CA).

Data and Resource Availability

The data sets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

RESULTS

Diabetes Causes a Reduction in Circulating PMo

We enumerated circulating leukocytes and determined the proportions of leukocyte subpopulations in WT diabetic mice (DM) at 5 months of diabetes and in age-matched nondiabetic WT control mice (C) (Supplementary Table 1*A*). Diabetes caused a reduction in the number of circulating WBC (WT-C: 7.4 ± 2.2 × 10^6/mL, WT-DM: 4.1 ± 0.9; $P < 0.0001$) and in the percentage of circulating lymphocytes (WT-C: 74.8 ± 6.5% of $CD45^+$, WT-DM: 64.4 ± 11.4%; $P = 0.015$) (Fig. 1*A*), while granulocytes (WT-C: 29.4 ± 9.9% of myeloid cells, WT-DM: 37.5 ± 11.2%) and total monocytes (WT-C: 55.4 ± 8.3% of myeloid cells, WT-DM: 48.5 ± 13.9%) were unchanged (Fig. 1*B*). Notably, diabetes caused a rearrangement of the monocyte subpopulations (Fig. 1*C*): intermediate monocytes were increased (WT-C: 13.2 ± 2.7% of total monocytes, WT-DM: 26.2 ± 7.4%; $P < 0.0001$), PMo were decreased (WT-C: 54.6 ± 13.9% of total monocytes, WT-DM: 37.1 ± 14.6%; $P = 0.008$), and inflammatory monocytes were unchanged (WT-C: 25.4 ± 15.1% of total monocytes, WT-DM: 25.9 ± 14.5%; P = NS). The levels of hyperglycemia showed an inverse correlation with the percentage of PMo ($r = -0.67$, $P = 0.03$) (Supplementary Fig. 2).

Taken together, these data show that diabetes reduces lymphocytes and PMo in the general circulation.

Diabetes Causes an Increase in PMo Firmly Adherent to Retinal Microvessels (Leukostasis)

To test whether in diabetes PMo may be recruited to specific microvascular regions, we counted PMo in perfused retinal vessels of WT and $NR4A1^{-/-}$ mice at 3 months of diabetes and in age-matched nondiabetic WT and $NR4A1^{-/-}$ control mice (Supplementary Table 1*B*). The FITC-$ConA^+$ cells remaining adherent to perfused retinal microvessels were

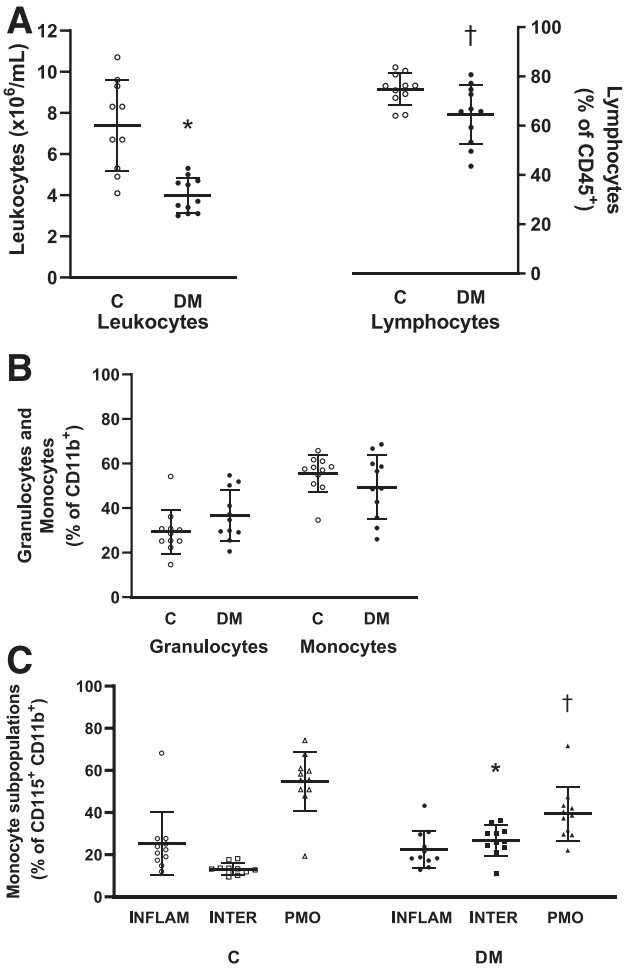

Figure 1—Circulating leukocyte number and subpopulations are changed at 5 months of diabetes. *A*: The absolute number of circulating leukocytes (left) and the percentage of lymphocytes (right) were decreased in WT-DM. Circulating leukocytes measured by hemocytometer were 7.4 ± 2.2 (mean ± SD) × 10^6/mL in WT-C (C) vs. 4.1 ± 0.9 ($P < 0.0001$) in WT–DM (DM). The percentage of lymphocytes measured by flow cytometry was 74.8 ± 6.5% of $CD45^+$ cells in C mice vs. 64.4 ± 11.4% in DM mice ($P = 0.015$). *$P < 0.0001$; †$P = 0.015$. Statistical analysis was performed using the Student *t* test. *B*: Granulocytes (left) and total monocytes (right) measured by flow cytometry and expressed as the percentage of circulating myeloid cells ($CD11b^+$) were not affected by diabetes. Granulocytes were 29.4 ± 9.9% (mean ± SD) in C mice vs. 37.5 ± 11.2% in DM mice (P = NS); total monocytes were 55.4 ± 8.3% in C mice vs. 48.5 ± 13.9% in DM mice (P = NS). Statistical analysis was performed using the Student *t* test. *C*: The inflammatory (INFLAM), intermediate (INTER), and PMo (PMO) subpopulations were selectively rearranged by diabetes. Monocyte subpopulations were measured by flow cytometry and expressed as percentage of total monocytes ($CD115^+$). Inflammatory were 25.4 ± 15.1% (mean ± SD) in C mice and 25.9 ± 14.5% in DM mice (P = NS), intermediate were 13.2 ± 2.7% in C mice vs. 26.2 ± 7.4% in DM mice ($P < 0.0001$), and PMo were 54.6 ± 13.9% in C mice vs. 37.1 ± 14.6% in DM mice ($P = 0.008$). *$P < 0.0001$ vs. intermediate monocytes in C mice, †$P = 0.015$ vs. PMo in C mice. Statistical analysis was performed using ANOVA, followed by post hoc testing with the Bonferroni test. *A*, *B*, and *C*: Error bars represent the mean ± SD; n = 10–11 mice/group for all measurements.

almost universally positive for the panleukocyte marker CD45 ($r = 0.96$, $P < 0.0001$) (Fig. 2*A* and *B*), indicating that they were WBC. To identify which proportion of adherent leukocytes were PMo, we used antibody to CD16.2. Among WBC, CD16.2 is expressed almost solely on PMo (granulocytes show one-tenth the level of expression in PMo) (17,18). We confirmed such specificity by comparing with flow cytometry circulating WBC from WT and NR4A1$^{-/-}$ mice, which lack PMo (Fig. 3*A*). Further, we performed CD16.2 immunohistochemistry in retinal vessels and observed that the antibodies to CD16.2 failed to stain any FITC-ConA^{+} cells in the retinal vessels of NR4A1$^{-/-}$ mice. Therefore, CD16.2^{+} cells in WT mice (control and diabetic) identify PMo (Fig. 3*B*).

We proceeded to enumerate PMo in retinal vessels (Fig. 3*C*). Consistent with previous observations in rats (19,20) and mice (21), retinal vessels of WT-DM mice showed a 60% increase in the number of adherent FITC-ConA^{+} cells compared with WT-C mice (WT-C: 58 ± 23 cells/retina, WT-DM: 93 ± 18; $P < 0.001$). Surprisingly, diabetic vessels showed an approximately fourfold increase in the absolute number of PMo, from 10 ± 4 cells/retina in WT-C to 37 ± 6 in WT-DM ($P < 0.0001$), representing a PMo increase from 19 to 43% of all firmly adherent FITC-ConA^{+} cells. The increased number of PMo (~27 cells/retina) could quantitatively account for the increase in the number of FITC-ConA^{+} cells found in the retinal vessels of the WT-DM.

We next tested whether leukostasis in diabetes was affected by the absence of PMo occurring in NR4A1$^{-/-}$ mice (Fig. 3*D*). In nondiabetic NR4A1$^{-/-}$ mice, the number of firmly adherent cells in perfused retinal vessels was the same as in nondiabetic WT mice. However, in diabetic NR4A1$^{-/-}$ mice, the number of adherent cells failed to increase, in contrast to the near doubling caused by diabetes in the WT mice and despite levels of hyperglycemia comparable to those of WT-DM mice.

These data show that *1*) PMo are a large portion of the cells adherent to retinal vessels, *2*) the number of adherent PMo increases in diabetes, and *3*) the extent of the increase in PMo matches precisely the increase in total adherent WBC caused by diabetes. Collectively, the observations indicate a prominent numerical contribution of PMo to the phenomenon of retinal leukostasis in early diabetes.

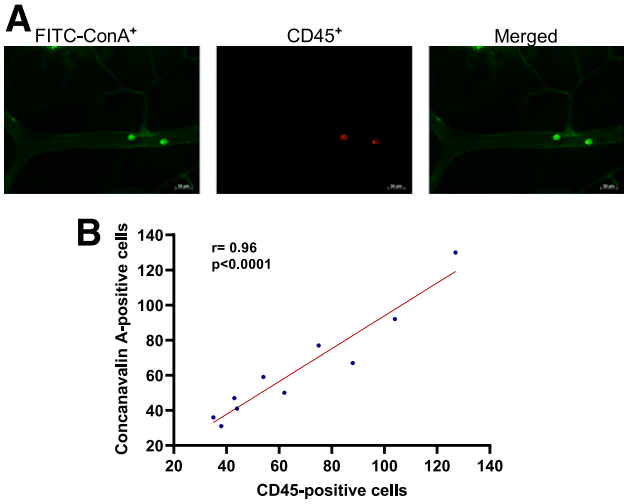

Figure 2—Adherent cells in retinal microvessels are CD45^{+}. *A*: ConA and CD45 colocalization in retinal microvessels of a WT-C mouse. Retinal microvessels immunostained for FITC-ConA (green, to identify adherent cells) and for Cy3-CD45 (red, to identify WBC) show colocalization of the two fluorochromes. *B*: Scatterplot and Pearson correlation between ConA^{+} and CD45^{+} cells ($r = 0.96$, $P < 0.0001$) show that FITC-ConA^{+} cells were almost universally positive for the panleukocyte marker CD45, indicating that they were WBC. Each point represents counts in the retinal microvessels of an individual mouse. Measurements were performed in mice from all four experimental groups (WT-C and DM, NR4A1$^{-/-}$-C and DM, $n = 10$).

In the Absence of PMo, Diabetic Microvascular Disease Is Increased in the Retina

Next, we tested whether the lack of PMo has an impact on the retinal capillary damage induced by diabetes. We studied mice with relatively short diabetes duration to capture potential acceleration of damage in the mice lacking PMo and to prevent or limit the confounding effect of any differential effect of aging on the retinal vessels in the WT and NR4A1$^{-/-}$ mice. At both 4 and 6 months of diabetes, the WT and NR4A1$^{-/-}$ mice were closely matched for severity of diabetes, as measured by insulin requirements (data not shown), and body weight, blood glucose, and HbA_{1c} at sacrifice (Supplementary Tables 1*C* and *D*). Four months of diabetes did not increase retinal vascular leakage, as detected by FA in WT or in NR4A1$^{-/-}$ mice, or change significantly the number of retinal acellular capillaries (AC), a well-established measure of diabetic retinal microangiopathy (22) (Supplementary Fig. 3*A* and *B*).

After 6 months of diabetes, there was still no leakage in fluorescein angiograms (Fig. 4*A*). As expected (14), diabetic WT mice had begun to show a small increase in the number of AC compared with nondiabetic WT controls (WT-C: 5 ± 1 per mm^2 retina, WT-DM: 7 ± 1; $P = 0.006$) (Fig. 4*B* and *C*). In contrast, in diabetic NR4A1$^{-/-}$ mice, the number of AC more than doubled compared with nondiabetic NR4A1$^{-/-}$ (NR4A1$^{-/-}$-C: 5 ± 1 per mm^2 retina, NR4A1$^{-/-}$-DM: 12 ± 3; $P < 0.001$). The number of AC in diabetic NR4A1$^{-/-}$ mice was also significantly greater than in the diabetic WT mice ($P < 0.0001$) (Fig. 4*B* and *C*). The similar number of AC in nondiabetic WT and nondiabetic NR4A1$^{-/-}$ mice at 8–10 months of age indicated a similar response to aging in the two genotypes.

The observation that absence of PMo is accompanied by an increase in AC in diabetes, but not in aging, provides support to our hypothesis that PMo are agents of vascular protection against diabetes.

Diabetes Primes a Protective Transcriptional Program in PMo

We thus investigated whether diabetes induces changes in the biosynthetic profile of PMo. We studied by next-generation sequencing the whole genomic transcriptome

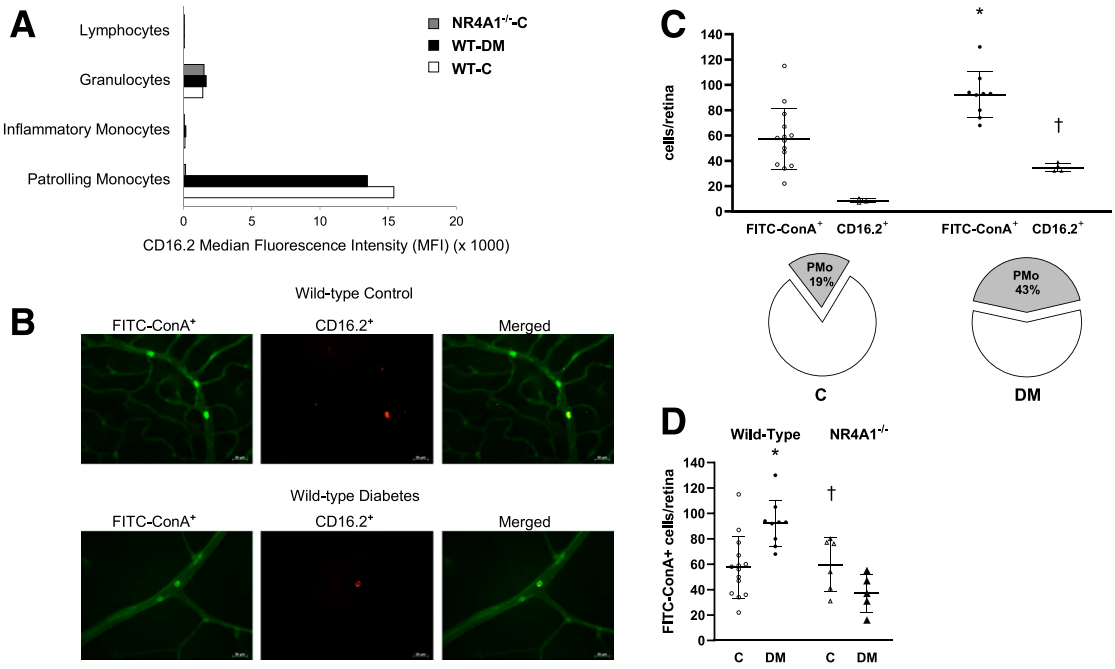

Figure 3—A subset of adherent cells in retinal microvessels is CD16.2 (FcgRIV)$^+$ and is increased at 3 months of diabetes. *A*: CD16.2 (aka FcgRIV) median fluorescence intensity measured by flow cytometry in circulating leukocyte subpopulations from WT-C, WT-DM, and NR4A1$^{-/-}$-C mice shows that among WBC, CD16.2 is expressed abundantly on PMo and to a minimal extent on granulocytes. This provides validation for the use of CD16.2 as an identifier of PMo in immunostaining studies of retinal vessels. *B*: Retinal microvessels immunostained for FITC-ConA (green, to identify leukocytes) and for Cy3-CD16.2 (red, to identify PMo) show colocalization of the two fluorochromes in a subset of adherent cells. *C*: PMo firmly adherent to retinal microvessels are increased at 3 months of diabetes. CD16.2$^+$ cells were counted in perfused retinal microvessels of WT-C (C) and of WT-DM mice (DM) with 3 months of diabetes. Top panel: the absolute number of FITC-ConA$^+$ cells (total leukocytes) was 58 ± 23 (mean ± SD) cells/retina in WT-C mice (n = 15) vs. 93 ± 18 in DM (n = 9; $P < 0.001$), and the absolute number of CD16.2$^+$ cells (PMo) was 10 ± 4 cells/retina in C mice (n = 4) vs. 37 ± 6 in DM (n = 5; $P < 0.0001$). Error bars represent the mean ± SD. Bottom panel: Full pie, total leukocytes (FITC-ConA$^+$ cells/retina); shaded portions, relative proportion of PMo (CD16.2$^+$ cells). The proportion of CD16.2$^+$ cells is increased in the DM mice to 43% of all firmly adherent FITC-ConA$^+$ cells vs. 19% in C mice. For each mouse, counts are the mean of the two eyes, performed by masked observers. *$P < 0.001$; †$P < 0.0001$ vs. control. Statistical analysis was performed using the Student t test. *D*: WBC firmly adherent to retinal microvessels do not increase at 3 months of diabetes in NR4A1$^{-/-}$ mice lacking PMo. While the number of FITC-ConA$^+$ cells per retina was increased in WT-DM mice (n = 9) compared with WT-C (n = 15), it did not change in NR4A1$^{-/-}$-DM (n = 5; FITC-ConA$^+$ cells = 37 ± 15) compared with NR4A1$^{-/-}$-C (n = 6; FITC-ConA$^+$ = 60 ± 21) (P = NS). Error bars represent the mean ± SD. *$P < 0.001$ vs. WT-C and NR4A1$^{-/-}$-DM; †$P = 0.02$ vs. WT-DM. Statistical analysis was performed using ANOVA for the four groups, followed by post hoc testing with the Bonferroni test.

of PMo sorted from WT mice at 5 months of diabetes and littermate controls (intermediate diabetes duration groups in Supplementary Table 2). The choice of diabetes duration took into account that structural effects of the lack of PMo on retinal vessels were evident in the NR4A1$^{-/-}$ mice at 6 months of diabetes. Hence, PMo in the WT mice might have activated a molecular protective program before that time. Principal component analysis—a procedure that identifies patterns in high-dimensional data—showed excellent separation between the control and diabetic samples (Fig. 5*A*). At 5 months of diabetes, there were 114 DEGs between PMo of diabetic and control mice at a fold-change of two or higher and FDR of ≤0.05 (Fig. 5*B*). DEGs listed with detailed function in Supplementary Table 3 show profound suppression of genes related to inflammation and immunity, and instead upregulation of genes indicative of a promigratory, vasculoprotective, and antiapoptotic program.

To further define the molecular processes enriched in PMo by diabetes, we analyzed enriched functional categories among detected genes using preranked GSEA. Downregulated genes in PMo sorted from diabetic mice compared with PMo from nondiabetic mice were significantly enriched with pathways related to inflammation, immune response, hemostasis, and apoptosis. Upregulated genes showed enrichment in gene sets related to complement, with upregulation of all three subunits of C1q involved in the classical pathway and uptake of apoptotic cells. Of note, complement inhibitors were instead downregulated (Fig. 5*C* and *D* and Supplementary Table 4).

To check results in independent sets of mice while learning about stability of gene expression changes induced by diabetes, we tested by qPCR the expression of selected genes in PMo from mice with 7 months of diabetes (long duration) (Supplementary Fig. 4). The pattern was consistent with the results obtained via RNA-Seq at 5 months of diabetes, which showed downregulation of proinflammatory cytokines and proadhesive molecules and downregulation of angiostatic CXCL4 that would

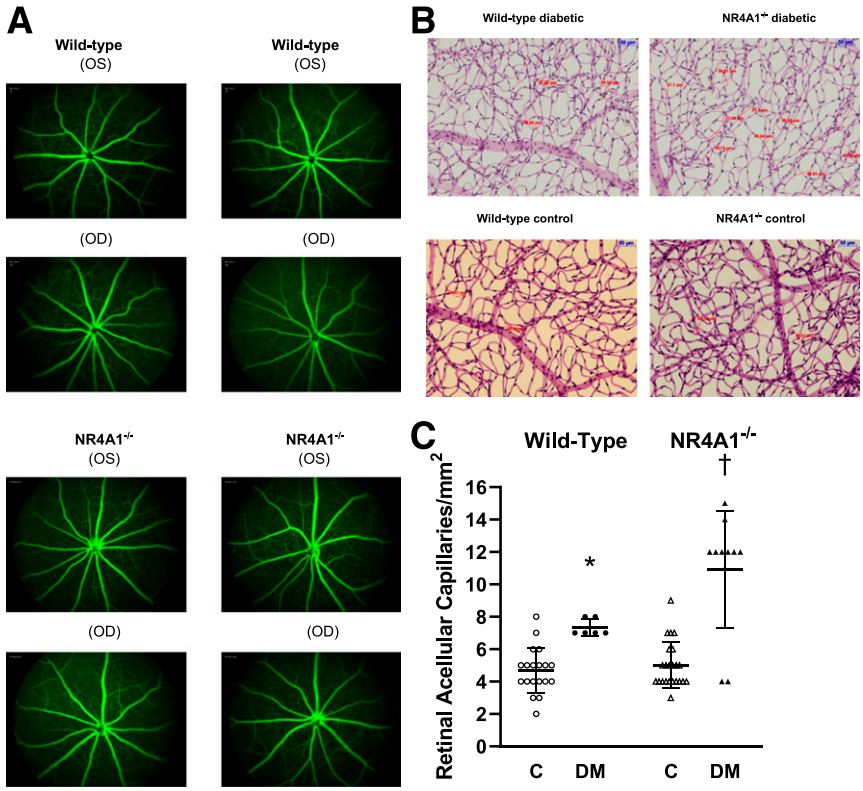

Figure 4—Retinal AC are increased at 6 months of diabetes in the absence of PMo. *A*: Representative retinal fluorescein angiograms performed at 6 months of diabetes in two WT (top panel) and two NR4A1$^{-/-}$ (bottom panel) mice. The images show absence of visible leakage. OD, right eye; OS, left eye. *B*: Retinal trypsin digests from one WT (left) and one NR4A1$^{-/-}$ (right) mouse at 6 months of diabetes show the development of AC. The AC are identified by red filling. The red numbers represent the length of the acellular segments, which must meet the criterion of being ≥40 μm. The two lower panels show AC in retinal trypsin digests from one WT-C and one NR4A1$^{-/-}$-C mouse at 18 months of age to document vascular integrity in aging in both genotypes. *C*: The absence of PMo augments the increase in retinal AC at 6 months of diabetes. The counts performed on retinal trypsin digests showed that AC per mm^2 retina were modestly increased by diabetes in WT mice (7 ± 1 [mean ± SD] in WT-DM [n = 6] vs. 5 ± 1 in WT-C [n = 19]; P = 0.006); and substantially increased by diabetes in NR4A1$^{-/-}$ mice lacking PMo (12 ± 3 in NR4A1$^{-/-}$-DM [n = 10] vs. 5 ± 1 in NR4A1$^{-/-}$-C [n = 22]); $P < 0.0001$ vs. NR4A$^{-/-}$-C and WT-DM). The identical counts in WT-C and NR4A1$^{-/-}$-C indicate that lack of PMo was not by itself a mechanism for capillary degeneration. Error bars represent the mean ± SD. Statistical analysis was performed using ANOVA for the four groups, followed by post hoc testing with the Bonferroni test. *P = 0.006 vs. WT-C; †$P < 0.0001$ vs. NR4A$^{-/-}$-C, WT-C, and WT-DM.

restore or enhance vascular endothelial growth factor activity, except for promigratory CXCR4 that was no longer upregulated. This prompted the testing of mice with 3 months of diabetes (short duration) to obtain a time course. At 3 months of diabetes, the major proinflammatory cytokines were not yet downregulated, but CXCR4 expression was already substantially increased.

Therefore, diabetes induces in PMo biosynthetic changes indicative of enhanced migratory, vasculoprotective, and housekeeping activities as well as profound suppression of genes related to inflammation and apoptosis. These changes are evident after 5 months of diabetes and persist at longer duration, except for the upregulation of promigratory CXCR4 that precedes the other changes and is lost at longer durations of diabetes.

DISCUSSION

The findings indicate that after a short duration of diabetes, PMo change their biosynthetic characteristics and are the cells preferentially recruited to the retinal vessels. Insofar as they express a promigratory and vasculoprotective biosynthetic program, and diabetic animals lacking PMo manifest enhanced obliteration of retinal capillaries, PMo can be viewed as protective against the hallmark lesion of diabetic retinal microangiopathy. Through these observations, diabetes joins other vascular pathologies—such as experimental glomerulonephritis (10), myocardial infarction (23), and atherosclerosis (24)—in which the study of PMo has uncovered unsuspected events that demand revision of prevailing paradigms.

Traditionally, increased leukostasis in diabetes has been interpreted as a sign of inflammation and attributed detrimental consequences. Schröder et al. (5) described for the first time an increased number of monocytes and granulocytes adherent to the retinal microvessels of diabetic rats and suggested that the phenomenon may play a key role in the pathogenesis of diabetic retinopathy causing capillary occlusion and endothelial damage. McLeod et al. (25)

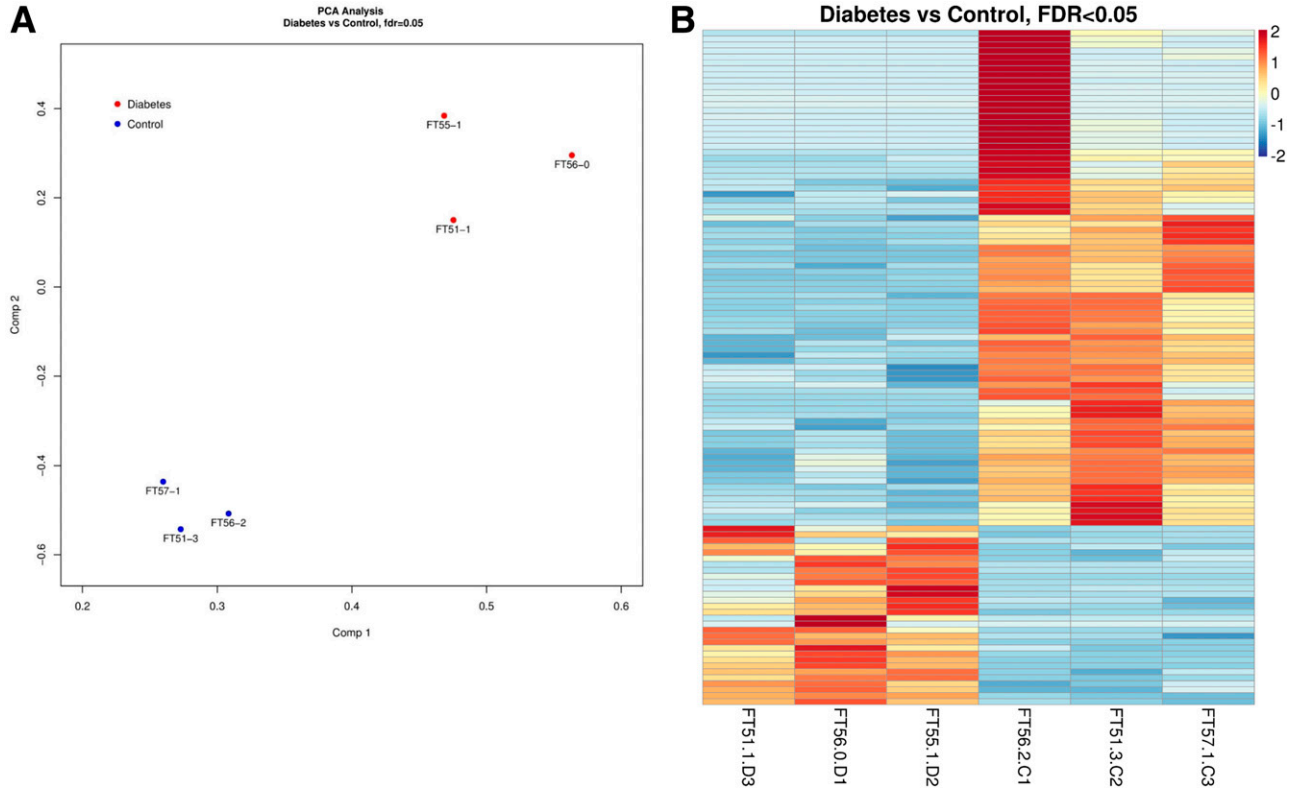

Figure 5—The gene expression of circulating PMo is changed at 5 months of diabetes. *A*: Principal component analysis of RNA-Seq data performed on three WT-C and three WT-DM mice at 5 months of diabetes (underneath each dot is the corresponding mouse identification code) shows that the main variability among circulating PMo with regard to the whole transcriptome is related to the presence of diabetes. Specifically, *1*) the three control samples appear very homogenous; *2*) the three diabetic samples appear slightly less homogenous than controls, as expected; and *3*) the two groups of control and diabetic samples are perfectly separated, showing a strong difference between the two groups. *B*: Heat map representing the expression levels of the differently expressed genes at FDR <0.05 and a fold-change of two or higher, as detected with RNA-Seq in circulating PMo, shows consistent difference between the control and the diabetic samples. Blue, low expression; red high expression. Each column represents results in one mouse. C1, C2, C3: RNA from three WT-C mice; D1, D2, D3: RNA from three WT-DM mice at 5 months of diabetes. *C*: GSEA curves show that genes with decreased expression in PMo sorted from DM compared with PMo from non-DM are significantly enriched with pathways related to inflammation, immune response, hemostasis, and apoptosis. Instead, genes with increased expression in PMo from DM are significantly enriched with pathways related to complement. Genes in the leading edge are indicated on the right side (decreased expression in diabetes) and on the left side (increased expression in diabetes) of the respective pathway. Enrichment plots were obtained from gene lists preranked according to log2 fold change in WT-DM mice at 5 months diabetes vs. WT-C. GSEA was performed on canonical pathway and ontology gene sets in the Molecular Signatures Database. *D*: Normalized enrichment scores (NES) of selected GSEA pathways enriched in PMo of mice at 5 months of diabetes. Barplots represent the NES of selected pathways from the indicated functional categories showing significant enrichment. Darkness of blue increases with NES absolute values.

reported in the retinal vessels of subjects with diabetes, with early or no retinopathy, increased expression of intercellular adhesion molecule 1 (ICAM-1), an endothelial molecule that enhances leukocyte adhesion, and Joussen et al. (4) reported that the inhibition of ICAM-mediated leukocyte adhesion reduced signs of retinopathy in mice with a long duration of diabetes. Increased leukostasis in the retinal microvasculature of diabetic animals was also shown in vivo using acridine orange leukocyte fluorography (26,27).

However, more recent studies have reported that the cells adherent to the retinal vessels of diabetic mice display antigenic features characteristic of PMo, even though the authors did not entertain explicitly that those cells may be PMo (28). Our demonstration of the numerical contribution of PMo to increased leukostasis in diabetes and of the accelerated vascular damage in diabetic animals lacking PMo now calls for a reinterpretation of the functional meaning of leukostasis: at least after short duration of diabetes, it is a mechanism to bring protective or healing influences to the cells of retinal vessels. This novel view is in line with the housekeeping-protective activities of PMo on microvessels in the kidney (8,10), in the cerebral cortex (29), and in the lung (30). If future studies in other models of experimental diabetes confirm PMo as the cell type preferentially retained by the endothelium in "diabetic leukostasis," then the phenomenon should be renamed "diabetic monostasis."

The NR4A1 orphan nuclear receptor is a master regulator of the differentiation and survival of PMo (12), and the NR4A1$^{-/-}$ mouse has been widely used as a model for

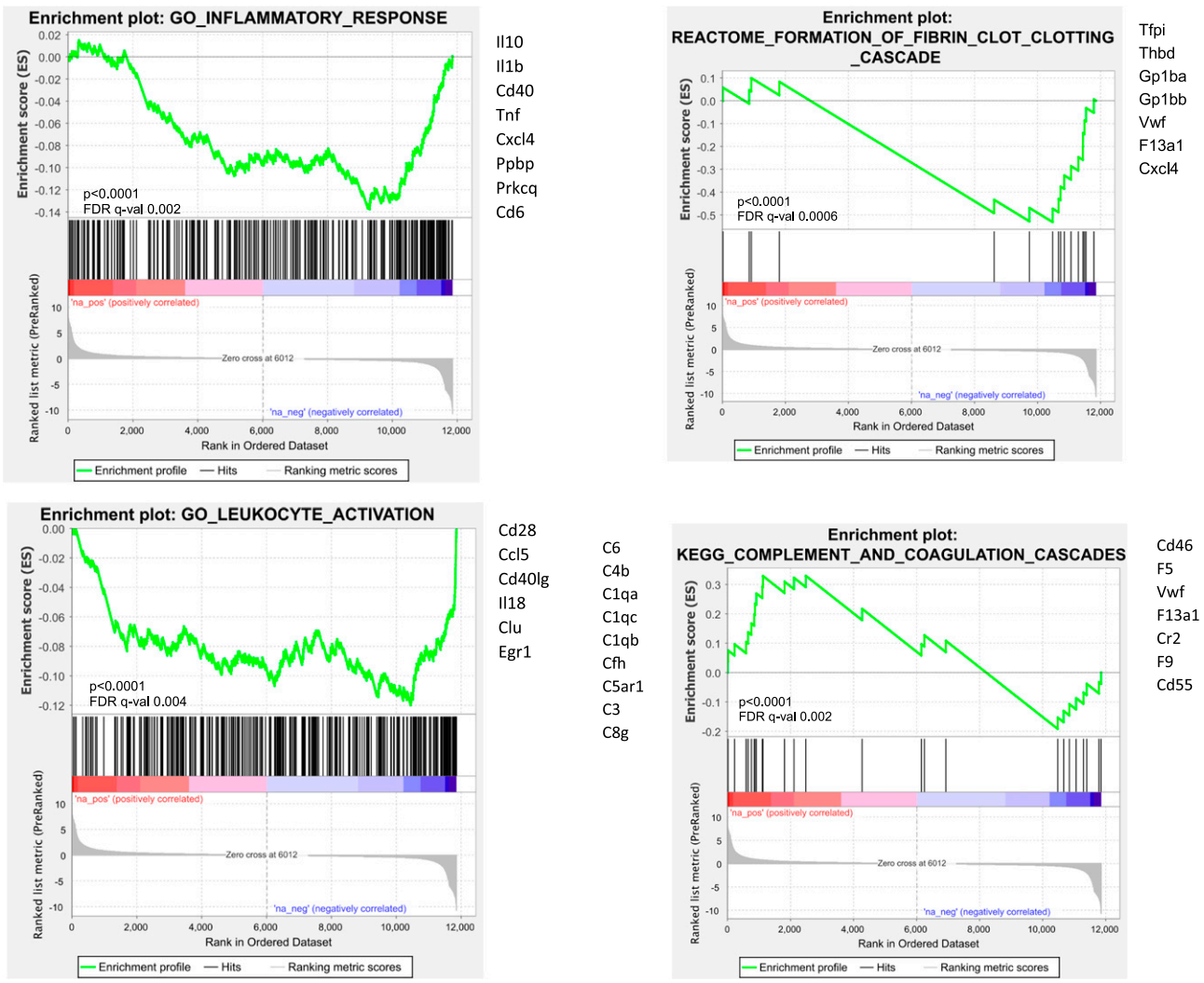

Figure 5—*Continued*.

lack of PMo in studies of micro- and macrovascular disease as well as of tumor metastasis (10,24,30). This study proved the suitability of the model for studies of diabetic microangiopathy. The NR4A1$^{-/-}$ mice did not become obese, did not develop spontaneous diabetes, and when diabetes was induced by streptozotocin, the severity of the hyperglycemia and the insulin requirement were similar in the WT and NR4A1$^{-/-}$ mice. As to potential effects of the NR4A1 deletion directly on the cells of the blood vessels, the NR4A1$^{-/-}$ mice have never been reported to have a developmental vascular or any phenotype (31), and the detailed examination performed in this study confirmed healthy-appearing retinal capillaries in mice at up to 18 months of age (see Fig. 4*B*). The absent effect of up to 6 months of diabetes on retinal vascular leakage, as detected by FA, mirrors the results obtained using dynamic contrast-enhanced MRI measurements (32) and precluded the possibility of testing whether the NR4A1 deletion reduced permeability (31). At this stage of the investigation, the accelerated microangiopathy in the diabetic NR4A1$^{-/-}$ mice cannot be solely attributed to the lack of PMo, insofar as the NR4A1 deletion present in other cell types may have provided unknown contributions. However, a direct and causal relationship is supported by concordant findings in two very different animal models. As in our NR4A1$^{-/-}$ mice, accelerated obliteration of retinal capillaries occurs in diabetic mice lacking the fractalkine receptor (CX3CR1) (33). In both rodents and humans, CX3CR1 is a defining antigenic and functional characteristic of PMo (6), critical for crawling (7) and for recruitment to the vascular endothelium in response to perturbations (10). Thus, mice lacking CX3CR1 are a model for functional lack of PMo, and the lack of PMo becomes the common link to increased vascular damage in two otherwise different animal models.

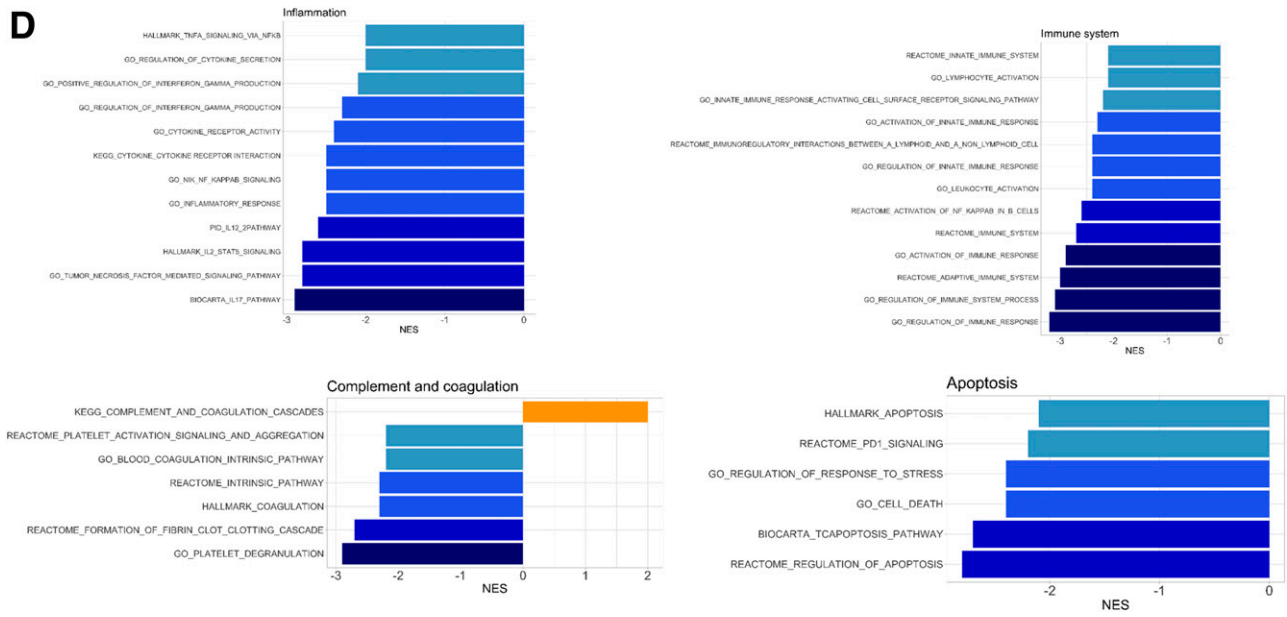

Figure 5—*Continued*.

To begin learning how PMo could protect vessels in diabetes, we studied the biosynthetic profile of circulating PMo. Circulating PMo can be expected to provide an informative representation of the biosynthetic activities of PMo adherent to vascular endothelium because at any given time, the latter represent one-third of the total circulating pool (or a greater proportion in the context of vascular pathologies) and are in constant exchange with the bloodstream (10). Recruitment of PMo to diabetic retinal vessels is likely to be triggered by subclinical endothelial injury, documented early in diabetes by gene expression studies showing the occurrence of oxidative stress and inflammation, vascular remodeling, activation of multiple mechanisms of apoptosis, and increased transforming growth factor-β signaling (34,35). The increased expression in diabetic PMo of CXCR4, the receptor that mediates chemotaxis toward a gradient of CXCL12, also known as stromal cell–derived factor 1 (SDF-1), suggests that their recruitment to retinal vessels occurs through the CXCL12-CXCR4 signaling pathway. Indeed, CXCR4 upregulation has been shown to be a critical event in the infiltration of PMo into tumors and causation of resistance to anti–vascular endothelial growth factor therapy (36,37). That diabetes had primed PMo toward migration was further documented by the substantial upregulation of the genes for *1*) Ppp1r9a-neurabin 1, which controls actin cytoskeleton organization; *2*) Shootin1, which couples F-actin and adhesive substrates as a clutch molecule to produce force for migration; and *3*) Claudin 1, the component of tight junctions that is increased in immune-activated leukocytes and also in patients with diabetes (see Supplementary Table 3). Once in contact with the vascular endothelium, diabetic PMo may exert protective actions via the increased expression of angiopoietin-like 4, a secreted molecule shown to induce the survival of endothelial cells in vitro and protect the integrity of the vascular network and endothelial junctions in animal models of myocardial infarction as well as ischemic stroke (38). The enrichment in gene sets related to complement, and in particular the upregulation of C1q that mediates the uptake of apoptotic cells (39,40), suggests that in diabetes, PMo use complement activation as a housekeeping mechanism for clearing vascular cells undergoing accelerated apoptosis (22). This proposes a new way of looking at the complement deposition that occurs early in diabetic retinal vessels, including in humans (41).

The profound downregulation of the expression of proinflammatory and immune genes that diabetes induced in PMo is unique, insofar as in all other types of leukocytes—from monocytes (42,43) to macrophages (44,45), granulocytes (46,47), and lymphocytes (48,49)—diabetes induces an inflammatory phenotype. For this reason, if the coordinated suppression in PMo of genes related to inflammation were found to reflect a cell type–specific response to metabolic abnormalities of diabetes, it would uncover a strikingly novel paradigm of cellular effects of hyperglycemia. But the downregulation may also result from interactions of PMo with other leukocytes in the context of leukostasis and/or signals emanating from the endothelium. In particular, the increased transforming growth factor-β signaling that occurs in retinal vessels after short diabetes duration (34) has anti-inflammatory effects and protects powerfully the integrity of retinal capillaries (35). The data on hand do not permit us to attribute a specific function to the suppression of genes related to inflammation below control levels, especially in

cells like the PMo that are known to normally produce noninflammatory cytokines (8,9).

By showing that protection and/or repair are elements of the natural history of diabetic retinal microangiopathy, our observations provide a new view of the early stages of diabetic retinopathy and new concepts for clinical translation. In particular, cells readily accessible, like circulating PMo, which we have documented to change gene expression in response to diabetes and likely in a time-dependent manner, could become useful probes for systematic monitoring of vascular status in patients. For example, CXCR4 upregulation in PMo might indicate that the endothelium has begun to suffer and is recruiting PMo in assistance, and loss of CXCR4 upregulation might signal the transition to a rapid progression of microangiopathy. There is suggestion of such a scenario in a clinical study in which we had observed CXCR4 downregulation in circulating endothelial progenitor cells carrying monocytic markers only when the cells were obtained from patients with diabetes with retinopathy, as opposed to patients free of retinopathy (50). Eventually, identification of the biosynthetic changes most critical to the protective role of PMo in diabetes may permit harnessing such functions through exogenous interventions.

Acknowledgments. The authors are grateful to Dr. C. Gerhardinger (Harvard Department of Stem Cell and Regenerative Biology) for her advice and assistance with RNA experiments, Dr. R. Nayar (Center for Systems Biology at Massachusetts General Hospital and Harvard Medical School) for her advice in planning the RNA-Seq procedure, and Dr. G. Zerbini and Prof. A. Giustina for continuous interest and encouragement.

Funding and Duality of Interest. This work was supported by the European Foundation for the Study of Diabetes (EFSD)/Boehringer Ingelheim European Research Programme in Microvascular Complications of Diabetes to F.T., the European Union's Horizon 2020 research and innovation programme under the Marie Skłodowska-Curie grant agreement No. 795877 to F.T., the Global Ophthalmology Award Program from Bayer to M.L., and National Institutes of Health National Eye Institute (grant R21 EY024108 to M.L.) and the core grant P30EY003790, and the Italian Ministry of Health (5×1,000). No other potential conflicts of interest relevant to this article were reported.

Author Contributions. F.T. and T.A.P. maintained the mouse colony, induced and managed diabetes in mice, and performed the flow cytometry and intracardiac perfusion experiments. F.T., T.A.P., and Z.D. performed the immunofluorescence experiments, trypsin digestions, and the counts of acellular capillaries. F.T. and M.L. designed the study, analyzed the data, and wrote the article. F.S. performed the real-time qPCR experiments. G.M.S. performed the bioinformatic analysis. F.T. and M.L. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

1. Hammes HP, Kerner W, Hofer S, Kordonouri O, Raile K, Holl RW; DPV-Wiss Study Group. Diabetic retinopathy in type 1 diabetes–a contemporary analysis of 8,784 patients. Diabetologia 2011;54:1977–1984

2. Gerhardinger C, McClure KD, Romeo G, Podestà F, Lorenzi M. IGF-I mRNA and signaling in the diabetic retina. Diabetes 2001;50:175–183

3. Frimmel S, Zandi S, Sun D, et al. Molecular imaging of retinal endothelial injury in diabetic animals. J Ophthalmic Vis Res 2017;12:175–182

4. Joussen AM, Poulaki V, Le ML, et al. A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 2004;18:1450–1452

5. Schröder S, Palinski W, Schmid-Schönbein GW. Activated monocytes and granulocytes, capillary nonperfusion, and neovascularization in diabetic retinopathy. Am J Pathol 1991;139:81–100

6. Geissmann F, Jung S, Littman DR. Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003;19:71–82

7. Auffray C, Fogg D, Garfa M, et al. Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior. Science 2007;317:666–670

8. Cros J, Cagnard N, Woollard K, et al. Human CD14dim monocytes patrol and sense nucleic acids and viruses via TLR7 and TLR8 receptors. Immunity 2010;33:375–386

9. Ingersoll MA, Spanbroek R, Lottaz C, et al. Comparison of gene expression profiles between human and mouse monocyte subsets. Blood 2010;115:e10–e19

10. Carlin LM, Stamatiades EG, Auffray C, et al. Nr4a1-dependent Ly6C(low) monocytes monitor endothelial cells and orchestrate their disposal. Cell 2013;153:362–375

11. Alexandraki KI, Piperi C, Ziakas PD, et al. Cytokine secretion in long-standing diabetes mellitus type 1 and 2: associations with low-grade systemic inflammation. J Clin Immunol 2008;28:314–321

12. Hanna RN, Carlin LM, Hubbeling HG, et al. The transcription factor NR4A1 (Nur77) controls bone marrow differentiation and the survival of Ly6C- monocytes. Nat Immunol 2011;12:778–785

13. Rojas M, Zhang W, Xu Z, et al. Requirement of NOX2 expression in both retina and bone marrow for diabetes-induced retinal vascular injury. PLoS One 2013;8:e84357

14. Kern TS, Tang J, Berkowitz BA. Validation of structural and functional lesions of diabetic retinopathy in mice. Mol Vis 2010;16:2121–2131

15. Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C, Lorenzi M. Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy. Diabetes 2004;53:2404–2411

16. Subramanian A, Tamayo P, Mootha VK, et al. Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles. Proc Natl Acad Sci U S A 2005;102:15545–15550

17. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV. FcgammaRIV: a novel FcR with distinct IgG subclass specificity. Immunity 2005;23:41–51

18. Biburger M, Aschermann S, Schwab I, et al. Monocyte subsets responsible for immunoglobulin G-dependent effector functions in vivo. Immunity 2011;35:932–944

19. Kinoshita N, Kakehashi A, Inoda S, et al. Effective and selective prevention of retinal leukostasis in streptozotocin-induced diabetic rats using gliclazide. Diabetologia 2002;45:735–739

20. Yamagishi S, Matsui T, Nakamura K, Takeuchi M, Imaizumi T. Pigment epithelium-derived factor (PEDF) prevents diabetes- or advanced glycation end products (AGE)-elicited retinal leukostasis. Microvasc Res 2006;72:86–90

21. McVicar CM, Ward M, Colhoun LM, et al. Role of the receptor for advanced glycation endproducts (RAGE) in retinal vasodegenerative pathology during diabetes in mice. Diabetologia 2015;58:1129–1137

22. Mizutani M, Kern TS, Lorenzi M. Accelerated death of retinal microvascular cells in human and experimental diabetic retinopathy. J Clin Invest 1996;97:2883–2890

23. Nahrendorf M, Swirski FK, Aikawa E, et al. The healing myocardium sequentially mobilizes two monocyte subsets with divergent and complementary functions. J Exp Med 2007;204:3037–3047

24. Quintar A, McArdle S, Wolf D, et al. Endothelial protective monocyte patrolling in large arteries intensified by Western diet and atherosclerosis. Circ Res 2017;120:1789–1799

25. McLeod DS, Lefer DJ, Merges C, Lutty GA. Enhanced expression of intracellular adhesion molecule-1 and P-selectin in the diabetic human retina and choroid. Am J Pathol 1995;147:642–653

26. Tamura H, Miyamoto K, Kiryu J, et al. Intravitreal injection of corticosteroid attenuates leukostasis and vascular leakage in experimental diabetic retina. Invest Ophthalmol Vis Sci 2005;46:1440–1444

27. Abiko T, Abiko A, Clermont AC, et al. Characterization of retinal leukostasis and hemodynamics in insulin resistance and diabetes: role of oxidants and protein kinase-C activation. Diabetes 2003;52:829–837

28. Serra AM, Waddell J, Manivannan A, Xu H, Cotter M, Forrester JV. CD11b+ bone marrow-derived monocytes are the major leukocyte subset responsible for retinal capillary leukostasis in experimental diabetes in mouse and express high levels of CCR5 in the circulation. Am J Pathol 2012;181:719–727
29. Michaud JP, Bellavance MA, Préfontaine P, Rivest S. Real-time in vivo imaging reveals the ability of monocytes to clear vascular amyloid beta. Cell Rep 2013;5:646–653
30. Hanna RN, Cekic C, Sag D, et al. Patrolling monocytes control tumor metastasis to the lung. Science 2015;350:985–990
31. Zhao D, Qin L, Bourbon PM, James L, Dvorak HF, Zeng H. Orphan nuclear transcription factor TR3/Nur77 regulates microvessel permeability by targeting endothelial nitric oxide synthase and destabilizing endothelial junctions. Proc Natl Acad Sci U S A 2011;108:12066–12071
32. Berkowitz BA, Roberts R, Luan H, Peysakhov J, Mao X, Thomas KA. Dynamic contrast-enhanced MRI measurements of passive permeability through blood retinal barrier in diabetic rats. Invest Ophthalmol Vis Sci 2004;45:2391–2398
33. Beli E, Dominguez JM II, Hu P, et al. CX3CR1 deficiency accelerates the development of retinopathy in a rodent model of type 1 diabetes. J Mol Med (Berl) 2016;94:1255–1265
34. Gerhardinger C, Dagher Z, Sebastiani P, Park YS, Lorenzi M. The transforming growth factor-beta pathway is a common target of drugs that prevent experimental diabetic retinopathy. Diabetes 2009;58:1659–1667
35. Dagher Z, Gerhardinger C, Vaz J, Goodridge M, Tecilazich F, Lorenzi M. The increased transforming growth factor-β signaling induced by diabetes protects retinal vessels. Am J Pathol 2017;187:627–638
36. Jung K, Heishi T, Khan OF, et al. Ly6Clo monocytes drive immunosuppression and confer resistance to anti-VEGFR2 cancer therapy. J Clin Invest 2017;127:3039–3051
37. Jung K, Heishi T, Incio J, et al. Targeting CXCR4-dependent immunosuppressive Ly6C^{low} monocytes improves antiangiogenic therapy in colorectal cancer. Proc Natl Acad Sci U S A 2017;114:10455–10460
38. Bouleti C, Mathivet T, Coqueran B, et al. Protective effects of angiopoietin-like 4 on cerebrovascular and functional damages in ischaemic stroke. Eur Heart J 2013;34:3657–3668
39. Navratil JS, Watkins SC, Wisnieski JJ, Ahearn JM. The globular heads of C1q specifically recognize surface blebs of apoptotic vascular endothelial cells. J Immunol 2001;166:3231–3239
40. Tran MTN, Hamada M, Jeon H, et al. MafB is a critical regulator of complement component C1q. Nat Commun 2017;8:1700
41. Zhang J, Gerhardinger C, Lorenzi M. Early complement activation and decreased levels of glycosylphosphatidylinositol-anchored complement inhibitors in human and experimental diabetic retinopathy. Diabetes 2002;51:3499–3504
42. Giulietti A, Stoffels K, Decallonne B, Overbergh L, Mathieu C. Monocytic expression behavior of cytokines in diabetic patients upon inflammatory stimulation. Ann N Y Acad Sci 2004;1037:74–78
43. Devaraj S, Glaser N, Griffen S, Wang-Polagruto J, Miguelino E, Jialal I. Increased monocytic activity and biomarkers of inflammation in patients with type 1 diabetes. Diabetes 2006;55:774–779
44. Kanter JE, Kramer F, Barnhart S, et al. Diabetes promotes an inflammatory macrophage phenotype and atherosclerosis through acyl-CoA synthetase 1. Proc Natl Acad Sci U S A 2012;109:E715–E724
45. Kraakman MJ, Murphy AJ, Jandeleit-Dahm K, Kammoun HL. Macrophage polarization in obesity and type 2 diabetes: weighing down our understanding of macrophage function? Front Immunol 2014;5:470
46. Karima M, Kantarci A, Ohira T, et al. Enhanced superoxide release and elevated protein kinase C activity in neutrophils from diabetic patients: association with periodontitis. J Leukoc Biol 2005;78:862–870
47. Hanses F, Park S, Rich J, Lee JC. Reduced neutrophil apoptosis in diabetic mice during staphylococcal infection leads to prolonged Tnfα production and reduced neutrophil clearance. PLoS One 2011;6:e23633
48. Jagannathan M, McDonnell M, Liang Y, et al. Toll-like receptors regulate B cell cytokine production in patients with diabetes. Diabetologia 2010;53:1461–1471
49. Belia S, Santilli F, Beccafico S, et al. Oxidative-induced membrane damage in diabetes lymphocytes: effects on intracellular Ca(2 +) homeostasis. Free Radic Res 2009;43:138–148
50. Zerbini G, Maestroni A, Palini A, et al. Endothelial progenitor cells carrying monocyte markers are selectively abnormal in type 1 diabetic patients with early retinopathy. Diabetes 2012;61:908–914