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Vascular Biology, Atherosclerosis, and Endothelium Biology

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

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We investigated the phenotype of cells involved in leukostasis in the early stages of streptozotocin-induced diabetes in mice by direct observation and by adoptive transfer of calcein-AM–labeled bone marrow–derived leukocytes from syngeneic mice. Retinal whole mounts, confocal microscopy, and flow cytometry *ex vivo* and scanning laser ophthalmoscopy *in vivo* were used. Leukostasis *in vivo* and *ex vivo* in retinal capillaries was increased after 2 weeks of diabetes (Hb A_{1c}, 14.2 ± 1.2) when either donor or recipient mice were diabetic. Maximum leukostasis occurred when both donor and recipient were diabetic. CD11b$^+$, but not Gr1$^+$, cells were preferentially entrapped in retinal vessels (fivefold increase compared with nondiabetic mice). In diabetic mice, circulating CD11b$^+$ cells expressed high levels of CCR5 (P = 0.04), whereas spleen (P = 0.0001) and retinal (P = 0.05) cells expressed increased levels of the fractalkine chemokine receptor. Rosuvastatin treatment prevented leukostasis when both recipient and donor were treated but not when donor mice only were treated. This effect was blocked by treatment with mevalonate. We conclude that leukostasis in early diabetic retinopathy involves activated CCR5$^+$CD11b$^+$ myeloid cells (presumed monocytes). However, leukostasis also requires diabetes-induced changes in the endothelium, because statin therapy prevented leukostasis only when recipient mice were treated. The up-regulation of the HMG-CoA reductase pathway in the endothelium is the major metabolic dysregulation promoting leukostasis. ***(Am J Pathol 2012, 181:719–727; http://dx.doi.org/10.1016/j.ajpath.2012.04.009)***

Diabetic retinopathy (DR) is an almost inevitable microvascular complication of prolonged elevated blood glucose in patients with both type 1 and type 2 diabetes mellitus.[1] The main site of pathological characteristics in DR is at the level of the endothelial cell, and the clinical features are considered to be the consequence of endothelial damage and retinal ischemia. Retinal ischemia itself is the result of capillary closure and has been attributed to various procoagulant mechanisms, including a fibrinogen-related prothrombotic tendency[2–4] and increased leukocyte-endothelial cell adhesion, which may all be present from an early stage of the disease, even before the clinical manifestations.[5,6]

The role of leukocytes in the pathogenesis of DR, and specifically in leukostasis with risk of capillary closure, has suggested that the condition may represent a form of low-grade inflammation.[5] Considerable experimental and clinical evidence has been gathered to demonstrate increased adhesion of leukocytes to the retinal endothelium.[7,8] In addition, there has also been evidence to show that reduction in leukocyte adhesion delays development of DR.[6]

Several questions derive from these observations: i) Is there selectivity in leukocyte subset adhesion? ii) Does

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elevated blood glucose itself induce leukocyte or endothelial cell activation or are these the result of some downstream effect of high blood glucose? iii) Are there specific molecules (chemokines) that target chemokine receptors on leukocytes and attract them to the retinal endothelium? Interestingly, chemokines, such as monocyte chemotactic protein-1 (or CCL2), have long been associated with diabetes and its complications.[9] More recently, polymorphisms in CCR5 have been associated with diabetes (specifically, the CCR5Δ32 polymorphism, which leads to CCR5 deficiency, was associated with better survival in type 2 diabetes, suggesting that CCR5 may be involved in diabetes complications).[10]

Initial seminal work by Schroder et al[8] indicated that both neutrophils and monocytic cells adhered to the retinal vasculature in an experimental model of DR. In addition, spontaneously diabetic monkey retinal vessels have contained more esterase-positive neutrophils in regions adjacent to areas of retinal ischemia.[11] More recently, extensive elegant studies by Adamis and colleagues[12] have shown that leukocytes adhere to the endothelium via up-regulation of adhesion molecules, such as leukocyte function–associated antigen-1 and intervascular and vascular cellular adhesion molecules (intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, respectively).[13–16] In addition, hyperglycemia can generate several mediators, including calpains in endothelial cells,[17] and can also lead to caspase-independent cell death.[18] In contrast, the release of specific growth factors from the ischemic retina is also important in this process, particularly the vascular endothelial growth factor (VEGF) isoform 164.[19] In particular, Muller cell VEGF release appears to be important in this process.[20] Adhesion molecule up-regulation on retinal vessels may also be directly due to poorly controlled blood glucose levels; for instance, short-term exposure to high concentrations of glucose can activate leukocytes[21] and receptor for advanced glycosylation end product expression on the endothelium, which subsequently can trigger leukocyte adhesion.[22] Indeed, this latter event is considered a more general mechanism for recruitment of leukocytes in inflammation and supports the notion that DR (and diabetes itself) is an inflammatory condition (previously described). Clinical studies in patients with diabetes have supported these findings, in which increased levels of soluble (shed) adhesion molecules in patients with DR have been shown and correlated with retinopathy severity.[23] More recently, we have also observed that lymphocytes may participate in this leukocyte activation process.[24]

The present study was undertaken to more closely investigate the relative contribution of different leukocyte subsets to leukostasis in diabetic retinal tissue and factors that may mediate their recruitment to the retina. Both *in vivo* and *ex vivo* experiments were performed using adoptive transfer of calcein-AM–labeled leukocytes with scanning laser ophthalmoscopy (SLO) and using retinal flat mount preparations with confocal microscopy and flow cytometry, respectively. This enabled us to evaluate the entire population of leukocytes involved in leukostasis within retinal vessels and to identify the subset of leukocytes involved in retinal vascular leukostasis in diabetic mice. In addition, we investigated the effects of rosuvastatin, an HMG-CoA inhibitor, on retinal vascular leukostasis.

Materials and Methods

Animals

C57BL/6 male mice, aged 10 to 12 weeks, were supplied by the Medical Research Facility (University of Aberdeen, Aberdeen, UK). All animals were housed according to the guidelines described in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Vision and Ophthalmic Research and also in accordance with requirements of the Animal License Act (UK). In addition, the procedures were approved by the Home Office Regulations for Animal Experimentation (UK). C57BL/6 mice weighing >30 g were made diabetic with a single i.p. injection of streptozotocin (STZ; 150 to 180 mg/kg) freshly dissolved in 0.9% sodium chloride w/v (Macoflex; MacoPharma, London, UK) and housed in conventional cages for 2 weeks. Glycemia control was performed on blood using a glucometer (Ascensia Spirit2; Bayer, Newbury, UK). After the animals were sacrificed, cardiopuncture was performed and red blood cell–glycated hemoglobin (Hb A_{1c}) was measured using a nonenzymatically Glyco-Tek affinity column (Helena Biosciences Europe, Newcastle upon Tyne, UK).

Preparation of Cell Suspensions from Healthy and Diabetic Tissues

Femurs and spleens were collected and placed in cold RPMI 1640 medium with L-glutamine (PAA Laboratories GmbH, Pasching, Austria). Bone marrow (BM) cells were flushed aseptically from the dissected femurs with cold RPMI 1640 medium through a 21-gauge needle (Thermo, Leuven, Belgium). The samples were passed through a 100-μm cell strainer (BD Biosciences Pharmingen, San Jose, CA) and washed with cold RPMI 1640 medium.

Blood samples were collected in 4% sodium citrate–coated syringes and vials, centrifuged at 800 $\times$ *g* for 10 minutes, and resuspended in RPMI 1640 medium. Red blood cells in BM and blood samples were lysed by ammonium chloride lysis buffer (0.83% NH_4Cl, pH 7.2). After lysis, cells were suspended in complete RPMI 1640 medium [1% sodium pyruvate (Flow Laboratories, Sutton, UK), 1% nonessential amino acids, 0.1% to 0.5% mercaptoethanol, 5% to 10% heat-inactivated fetal calf serum, and 2% penicillin-streptomycin (Gibco, BRL Technologies, Paisley, UK)] for further analysis.

Eyes were collected, placed in RPMI 1640 medium, and then dissected. Retinal samples were digested in 0.442 U/mL collagenase A plus 0.5 U/mL dispase II (Roche, Penzberg, Germany) per mouse retina for 180 minutes at 37°C. After digestion, retinal cell samples were passed through a 100-μm strainer and finally resuspended in complete RPMI 1640 medium.

Immunohistological Characteristics of Retinal Whole Mounts

Retinal whole mounts were prepared as previously described.[25] Some samples were prepared by dissection of the fixed enucleated globes without prior infusion. Single- and double-antibody staining protocols were followed, as previously described.[25] Mice were perfused i.v. with 0.1 mL of 2% Evans blue dye (Sigma, Poole, UK) after an SLO examination (see later), before sacrifice. The eyes were immediately enucleated and fixed in 2% paraformaldehyde. Retinas were dissected, stained with appropriate antibodies (as detailed later), and flat mounted using Vectashield (Vector Laboratories Inc., Burlingame, CA) fluorescence anti-fading medium for confocal evaluation of retinal leukocyte entrapment.

Primary antibodies used were as follows: purified rat anti-mouse CD3, Gr-1 (BD Biosciences Pharmingen), CD11b and IgG2a (Serotec, Oxford, UK), and biotin-conjugated rat anti-mouse CD45 and IgG2bk monoclonal antibodies (BD Biosciences Pharmingen). Blood vessels were stained with fluorescein isothiocyanate–conjugated antibody to platelet endothelium cellular adhesion molecule-1 (CD31) (BD Biosciences Pharmingen). After 1 hour of fixation in 2% paraformaldehyde (Sigma-Aldrich, Steinheim, Germany), eyes were dissected in PBS, followed by permeabilization of the retinas with 0.3% Triton X-100 (Sigma-Aldrich) plus 1% bovine serum albumin (Sigma-Aldrich) for 2 hours. After blocking the tissues in 10% rat serum, the whole mounts were incubated with primary antibodies 1:100 overnight, washed in PBS, and then further incubated with rabbit anti-rat biotinylated 1:100 or fluorescein isothiocyanate–conjugated antibody 1:50 (Dako Cytometry, Stockport, UK). Finally, fluorescein streptavidin 1:50 (Vector Laboratories Inc., Burlingame, CA) and/or tetramethyl rhodamine isothiocyanate-conjugated streptavidin 1:100 or 1:200 (Jackson Immunoresearch Laboratories Inc., West Grove, PA) fluorochromes were added.

Adoptive Transfer of BM-Derived Monocytes

For *in vivo* SLO experiments (see later), spleen leukocytes (2×10^6 cells/mL) in complete RPMI 1640 medium were incubated with 40 μg/mL calcein-AM (Molecular Probes, Leiden, The Netherlands) at 37°C for 30 minutes. Cells were washed and assessed for viability by dye exclusion. Leukocyte fluorescence was confirmed by microscopy (Olympus, London, UK), and cells were suspended at 1×10^8/mL in RPMI 1640 medium before adoptive transfer. Leukocytes were adoptively transferred between donor and recipient diabetic and nondiabetic (control) mice in various combinations, as follows: group 1, control; group 2, diabetic (donor only); group 3, diabetic (recipient only); group 4, diabetic (recipient and donor); group 5, control, rosuvastatin treated; group 6, diabetic (donor and recipient, rosuvastatin treated); group 7, diabetic (recipient only, rosuvastatin treated); group 8, diabetic (donor only, rosuvastatin treated, prevention group); group 9, same as for group 6, plus mevalonate treated; and group 10, same as for group 6, but rosuvastatin administered after diabetes established, intervention group.

For *ex vivo* tracking of labeled leukocytes in retinal flat mounts, BM cells (2×10^7) were labeled with 10 μg/mL of calcein-AM in 0.1% bovine serum albumin/PBS for 30 minutes at 37°C. After thorough washes, the cells were resuspended in PBS for i.v. injection. One hour after injection, the mice were sacrificed, the eyes were enucleated, and the retinas were dissected, fixed, permeabilized, and prepared as previously described.

In Vivo *Cell Tracking Using SLO and Image Analysis*

Recipient mice were anesthetized with 0.4 mL/kg fentanyl-fluanisone (Hypnorm; Janssen-Cilag Ltd, Brussels, Belgium) i.m. and with 1 mL/kg diazepam (Phoenix Pharmaceuticals Ltd, Gloucester, UK) i.p., producing deep anesthesia for 1 hour. Pupils were dilated with 0.5% (w/v) tropicamide (Minims; Chauvin Pharmaceuticals Ltd, Essex, UK). A hard contact lens [clear polymethylmethacrylate: refractive index, 1.51; radius of curvature, 1.7 mm; diameter, 3.2 mm (Cantor & Nissel, Northamptonshire, UK)] was placed on the mouse cornea to obtain a clear view of the fundus. Viscotears were applied to generate smooth contact between the eye and the lens. Animals were kept immobile and maneuvered for examination using a movable tripod. A low dose (0.1 mL of 0.1% sodium fluorescein; Sigma, Poole, UK) was injected i.v. via the tail vein to outline the retinal vasculature, and animals were positioned for optimal viewing of the ocular fundus before i.v. infusion of 1×10^7 in 100 μL of calcein-AM–labeled leukocytes.

Equivalent fundus areas of each mouse were examined using a custom-built SLO at the University of Aberdeen School of Medicine.[26] In each animal, the nasal fundus adjacent to the optic disc was chosen and the animal was positioned so that a retinal artery and vein could be viewed. An argon laser (wavelength, 488 nm) was used to excite, and a 515-nm barrier filter was used to detect fluorescence. Real-time images were recorded continuously for the first 30 minutes after administration of fluorescent leukocytes into recipient mice using a DVD recorder (Sharp, Uxbridge, UK), as previously described.[27] When analyzing the DVD images, leukocytes were classified as either free flowing or stopping/adhering for a period of 1 to 2 seconds (transient attachment), 3 to 20 seconds (prolonged attachment), or >20 seconds (permanent adhesion), and the total number of cells stopping for >1 second was calculated.

Flow Cytometry

Single-cell suspensions were prepared as previously described, and cell viability was checked by trypan blue (VWR International Ltd, Leicester, UK) staining using a hemocytometer. BM and spleen (1×10^6 cells/test), blood (5×10^5 cells/test), and retina (single retina/test) cells were resuspended in 10% rat and mouse serum (Serotec, Oxford, UK) to block nonspecific fluorescence

and then incubated with a 1:50 dilution of antibody cocktail for 30 minutes at 4°C. The antibody panel used was CD45-allophycocyanin, CD11b-PerCpCy5.5, and CCR5-phosphatidylethanolamine monoclonal rat anti-mouse (BD Biosciences Pharmingen) and CCR2 and fractalkine chemokine receptor (CX3CR1) rabbit biotinylated with phosphatidylethanolamine-Cy7 (Abcam, Cambridge, UK). A biological positive control of CCR5 expression was performed by *in vitro* stimulation of splenocytes with concanavalin-A and IL-2 incubated at 37°C and 5% CO_2 for 3 days. Cellular staining was detected using a BD Biosciences LSR flow cytometer (BD Biosciences Pharmingen) with Ar and HeNe lasers. A total of 50,000 events were captured for each sample. Flow Jo cell analysis program (Tree Star, Inc., Ashland, OR) was used to analyze flow cytometric data, where gates and instrument settings were set according to forward and side scatter characteristics and populations gated to exclude dead or clumped cells.

Statistical Analysis

Data for the *ex vivo* analysis are expressed as mean ± SD. Statistical significance was determined by using an unpaired Student *t*-test, one or two tailed, as appropriate. The correlation was calculated using Spearman's test. Data for the *in vivo* analysis are presented as the mean ± SE. They were subjected to Bartlett's test for homogeneity of variances before analysis of variance. Where significance was reached ($P < 0.05$), between-group differences were established using the Newman-Keuls multiple-comparison test. All calculations were made using a standard software package (Prism3; Graphpad, San Diego, CA).

Results

We previously reported that the retinal changes in the STZ-induced mouse model of early-onset DR (2 weeks) manifested predominantly as capillary leukostasis.[28] A single injection of STZ (150 to 180 mg/kg) induced weight reduction, glycosuria (data not shown), and elevated blood glucose, with corresponding increased Hb A_{1c} levels (see Supplemental Tables S1 and S2 at *http://ajp.amjpathol.org*). A separate set of animals was used for the *in vivo* analysis, with similar results (data not shown). Neither weight loss nor plasma glucose levels were significantly altered by rosuvastatin treatment alone or with mevalonate cotreatment (see Supplemental Table S2 at *http://ajp.amjpathol.org*). Total plasma cholesterol and triglyceride concentrations were not significantly altered by STZ-induced diabetes or treatment with rosuvastatin (see Supplemental Table S2 at *http://ajp.amjpathol.org*).

Effect of Diabetes on in Vivo *Leukocyte-Endothelial Cell Interactions in the Retinal Microcirculation*

Adoptive transfer of leukocytes was performed in various donor-recipient combinations, as described in *Materials and Methods*. Two weeks of hyperglycemia was sufficient to cause a significant increase in the number of adoptively transferred leukocytes adhering to the retinal vasculature, irrespective of whether the donor, recipient, or both were diabetic [group 1 (control) versus groups 2 and 3 ($P < 0.01$) and 4 ($P < 0.001$); Figure 1A]. The total number of leukocytes adhering to the retinal vasculature was always significantly higher when the recipient mice were diabetic [diabetic donor (Dd) versus diabetic recipient (Dr), $P < 0.001$; control donor (Cd) versus Dr, $P < 0.01$], suggesting that the effect of diabetes on the endothelium was a stronger risk factor for leukocyte adhesion than the effects of diabetes on the leukocytes.

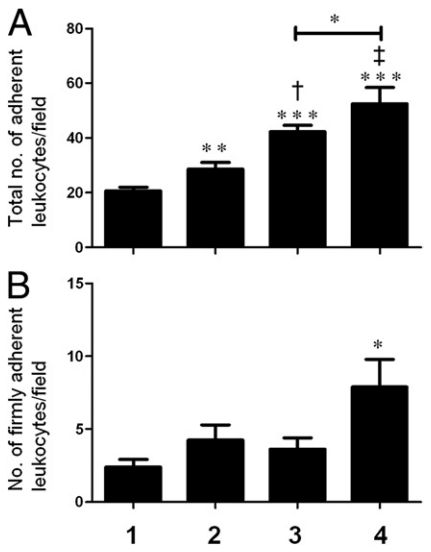

Figure 1. Effect of 2 weeks' STZ-induced diabetes on retinal leukocyte adhesion *in vivo*, assessed by SLO. Leukocytes isolated from the spleens of donor mice are labeled with the fluorescent marker, calcein-AM, adoptively transferred into recipient mice via the tail vein, and are tracked in the retinal circulation using SLO. Groups are defined as follows: 1, Cd¡Cr; 2, Dd¡Cr; 3, Cd¡Dr; 4, Dd¡Dr. C, control; D, diabetic; d, donor; r, recipient. **A:** The total number of calcein-AM–labeled leukocytes stopping in the retinal circulation for >1 second during a 30-minute observation period after cell transfer is shown for each experimental group. The following comparisons are significantly different: $^{*}P > 0.05$ for Cd→Dr versus Dd→Dr; $^{**}P < 0.01$ versus Cd→Cr; $^{***}P < 0.001$ versus Cd→Cr; $^{\dagger}P < 0.01$ versus Dd-Cr; $^{\ddagger}P < 0.001$ versus Dd-CR. **B:** The number of calcein-AM–labeled leukocytes stopping in the retinal circulation for >20 seconds (firm adhesion) during a 30-minute observation period. The following comparisons are significantly different: $^{*}P < 0.05$ versus Cd→Cr.

An analysis of the duration of adhesion (stasis) showed that most of the increase in leukocyte adhesion was due to transient attachments (duration, 1 to 2 seconds) to the retinal vasculature (control = 57.3% of cells adhering for group 1 versus diabetic = 68.5%, 72.1%, and 69.3% of cells adhering for groups 2, 3, and 4, respectively; Figure 1A), whereas firm, prolonged adhesion (>20 seconds) was less frequent (control = 11.6% of cells adhering for group 1 versus diabetic = 14.7%, 8.5%, and 15.1% of cells adhering for groups 2, 3, and 4, respectively; Figure 1B).

Retinal Vessels of Diabetic Mice Contain More $CD11b^{+}$ Cells

Retinal whole mounts were triple stained using CD31 to reveal the vasculature and various leukocyte markers to

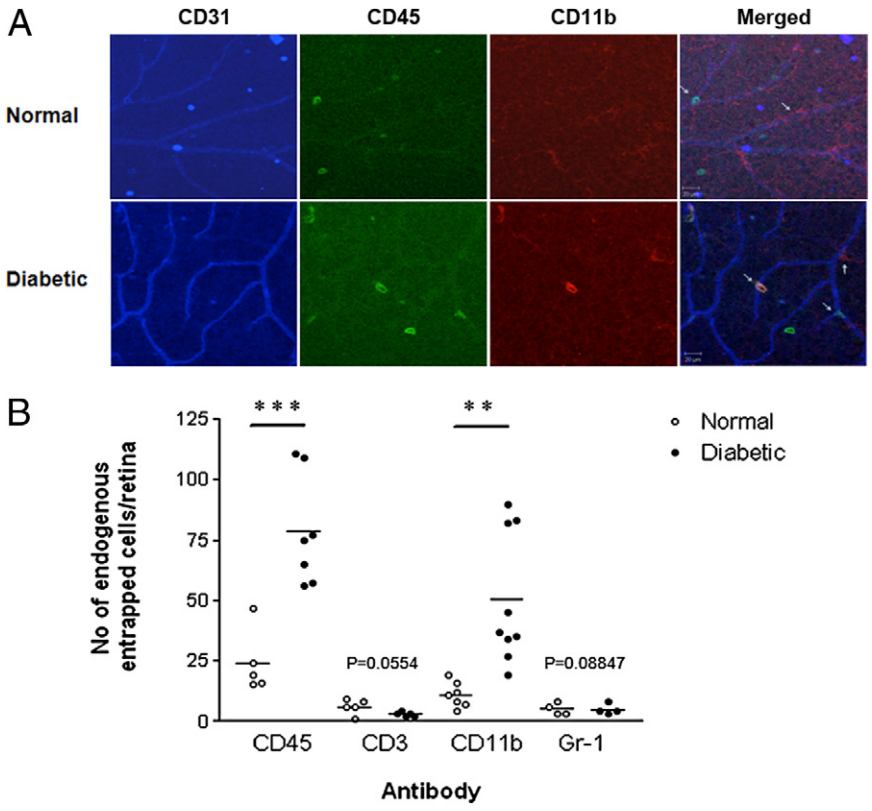

Figure 2. Diabetic retinal vessels contain increased numbers of CD11b$^+$ cells. **A:** Triple-immunofluorescence staining of murine healthy and diabetic retinal whole mounts with antibody to CD31-APC (blue), CD45–fluorescein isothiocyanate (FITC) (green), and CD11b–tetramethyl rhodamine isothiocyanate (red). **Arrows**, intravascular CD11b$^+$ cells. Scale bar = 20 μm. Original magnification, ×40. **B:** Increased numbers of intravascular CD11b$^+$ cells in diabetic retinas. Quantification of healthy and diabetic intravascular leukocytes per retina shows that the increased number of CD45$^+$ cells is attributable to a CD11b$^+$ myeloid cell subpopulation (approximately 50 cells per diabetic retina) but not to Gr-1$^+$ cells or CD3$^+$ T cells. Data are expressed as mean ± SD ($n \geq 4$), and the unpaired Student's *t*-test is used to calculate the significance. $^{**}P < 0.01$, $^{***}P < 001$.

identify leukocyte subsets, as described in *Materials and Methods*. Retinal vessels in 2-week-old diabetic mice contained increased numbers of CD45$^+$ leukocytes. In addition, there appeared to be a selective increase in the numbers of intravascular CD11b$^+$ cells, whereas GR-1$^+$ and CD3$^+$ T cells were not significantly increased. Few, if any, CD11b$^+$ leukocytes appeared to be situated extravascularly in the retinal parenchyma (Figure 2A). Quantitative analysis indicated that the increase in CD45$^+$ leukocytes in the retina of diabetic mice was statistically significant ($P = 0.0005$) and was predominantly attributable to increased numbers of CD11b$^+$ cells ($P = 0.002$; Figure 2B).

To confirm the source of the adherent retinal CD11b$^+$ cells as circulating BM-derived cells, adoptive transfer of calcein-AM–labeled BM-derived cells was performed from healthy to healthy mice (control) and from diabetic to diabetic mice. In the first set of experiments, retinas were flat mounted after perfusion of the circulation with Evan's blue dye to delineate the retinal vasculature. Figure 3 shows that the labeled leukocytes remained within the vasculature, that there were more intravascularly en-

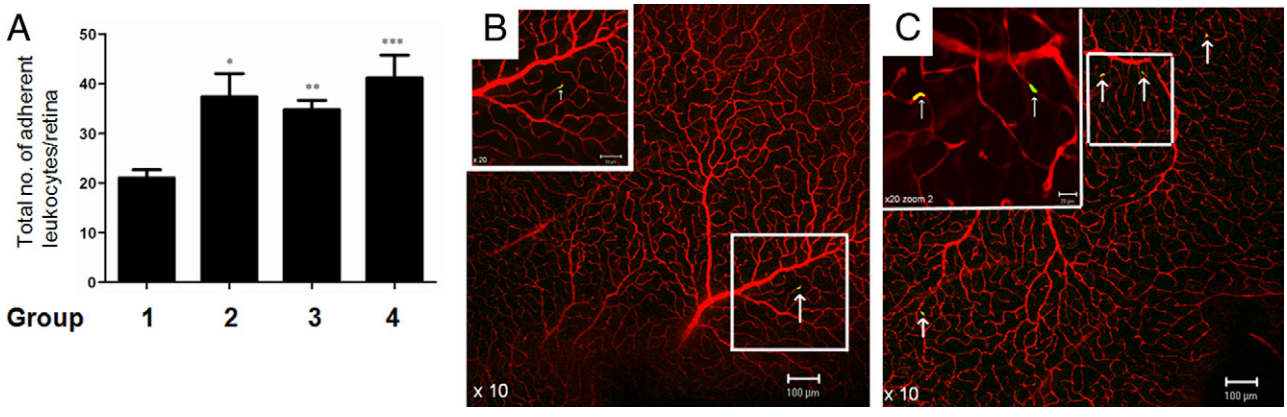

Figure 3. Effect of 2 weeks' STZ-induced diabetes on retinal capillary leukocyte entrapment in a mouse model of diabetes evaluated by confocal microscopy of retinal whole mount preparations. **Arrows**, intravascular adoptive transfer spleen leukocytes. **A:** The number of calcein-AM–labeled intravascular adherent leukocytes per retina is plotted in histograms for each group. Significant differences between the groups are observed as indicated: $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus group 1. **B** and **C:** Representative sample confocal images of retinal whole mounts: control mouse (group 1) (**B**) and diabetic mouse (group 4) (**C**). Groups are defined as follows: 1, Cd→Cr; 2, Dd→Cr; 3, Cd→Dr; 4, Dd→Dr. C, control; D, diabetic; d, donor; r, recipient.

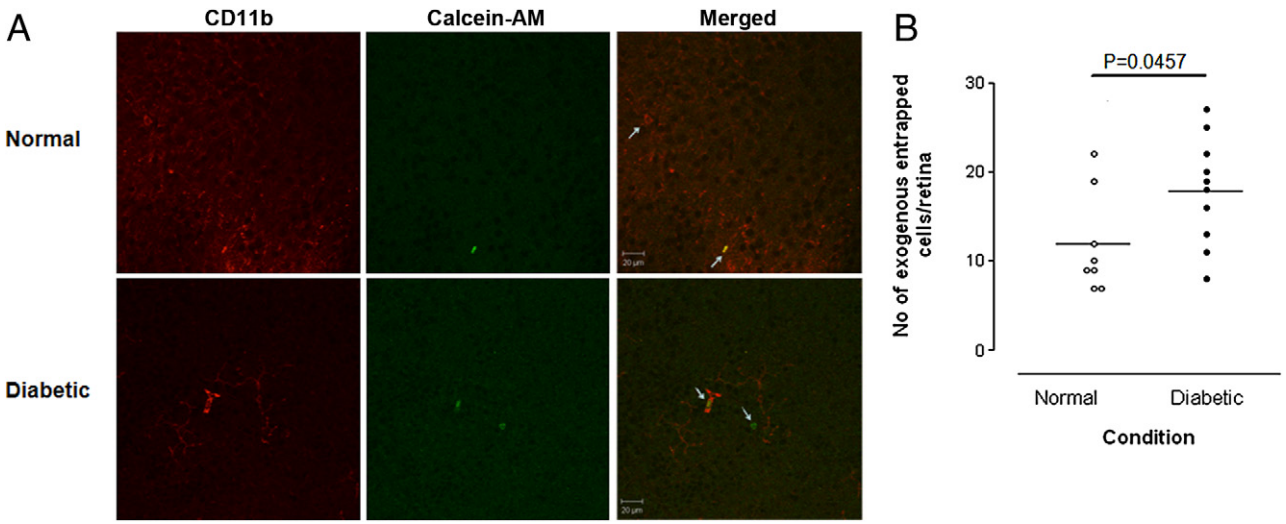

Figure 4. Increased numbers of adoptively transferred CD11b$^+$ BM-derived cells are trapped in retinal vessels from diabetic mice. **A:** Single-immunofluorescence staining of healthy and diabetic retinal whole mounts with antibody to CD11b–tetramethyl rhodamine isothiocyanate (red) after adoptive transfer of BM calcein-AM–labeled cells (green). **Arrows**, yellow dual-stained/labeled cells with CD11b and calcein-AM; or green labeled BM-derived cells. Scale bar = 20 μm. Original magnification, ×40. **B:** Quantification of retinal vessel-adherent adoptively transferred BM cells in healthy and diabetic mice. Data are expressed as mean ± SD ($n \geq 8$), and the one-tailed unpaired Student's *t*-test is used to calculate the significance.

trapped leukocytes in mice with diabetes than in healthy control mice, and that none of the labeled cells were detected within the neural retinal tissue, indicating that within this time frame (1 hour after transfer), no extravasation of cells occurred. In a second series of mice, retinas of the recipient mice were flat mounted 1 hour after adoptive transfer of calcein-AM–labeled BM-derived cells and stained with anti-CD11b antibody. Figure 4A shows that the calcein-AM–positive cells also stained for CD11b and that more double-positive cells were found in retinas from diabetic mice ($P = 0.046$; Figure 4B).

Circulating CD11b$^+$ Cells in Diabetic Mice Express Higher Levels of CCR5 and CX3CR1 than Cells from Healthy Mice

Chemokine polymorphisms have been associated with the complications of diabetes,[10] and there may be a genetic susceptibility that correlates with leukocyte-endothelial cell interactions in such circumstances. This may, however, be restricted to certain subset populations,[29] although, interestingly, CCR5$^{-/-}$ nonobese diabetic mice developed a more severe form of diabetes, whereas CCR2$^{-/-}$ mice were less affected.[30] We, therefore, investigated, using flow cytometry, whether there were changes in chemokine receptor expression in circulating CD11b$^+$ leukocytes that might have indicated an increased tendency to home to peripheral tissues. Our data revealed that there was a greater percentage of CCR5$^+$CD11b$^+$ cells in the blood ($P = 0.04$) and spleen ($P = 0.002$) from 2-week-old diabetic mice (Figure 5, A and B) compared with healthy control mice. However, although there was a trend toward higher CCR5 expression in retinal cells of diabetic mice compared with healthy mice, this was not statistically significant. Interestingly, the numbers of CCR2$^+$CD11b$^+$ cells were only increased in the spleen ($P = 0.001$) but not in the blood or in the retina of diabetic mice compared with nondiabetic mice (Figure 5, A and C). In contrast, CX3CR1 expression was up-regulated on retinal ($P = 0.05$) and spleen ($P = 0.0001$) CD11b$^+$ cells from diabetic mice (Figure 5, A and D). Also, the geometric mean fluorescence of CX3CR1 was up-regulated on retinal CD11b$^+$ cells isolated from diabetic mice ($P = 0.013$, Table 1). Increased CCR5 expression on circulating CD11b$^+$ cells correlated weakly with the level of hyperglycemia ($P = 0.05$, $r = 0.276$) (data not shown).

Effect of Rosuvastatin on in Vivo *Retinal Intravascular Leukocyte Adhesion in Diabetic Mice*

The diabetes-induced increase in retinal leukocyte adhesion was abolished by rosuvastatin treatment, using both prevention (group 6, 18.8 ± 2.3) and intervention (group 10, 19.6 ± 1.0) protocols of donor and recipient mice ($P < 0.001$; Figure 6) (see *Materials and Methods* and figure legends for details of group categories). The drug itself had no effect on basal levels of leukocyte adhesion, because the total number of adoptively transferred leukocytes stopping in the retinal circulation of rosuvastatin-treated control mice did not differ significantly from untreated control mice (group 1 versus group 5, 20.7 ± 2.9 versus 20.6 ± 1.4; $P > 0.05$; Figure 6). Rosuvastatin treatment of recipient animals only inhibited diabetes-induced leukocyte adhesion (group 7 versus group 4, 20.4 ± 3.0 versus 48.6 ± 6.6; $P < 0.001$). However, leukocytes from rosuvastatin-treated diabetic mice were almost equally as adherent as leukocytes from untreated diabetic mice (group 8 versus group 4, 36.6 ± 4.1 versus 48.6 ± 6.6; Figure 6), despite some reduction in overall events. Interestingly, cotreatment with mevalonate abrogated the effect of rosuvastatin on retinal leukocyte adhesion in diabetic mice, as shown by the absence of a significant difference between this group (group 9) and

Figure 5. Increased expression of CCR5, CCR2, and CX3CR1 chemokine receptors on diabetic CD11b$^+$ cells. **A:** Representative dot plots of CCR5, CCR2, and CX3CR1 cell surface expression on healthy and diabetic CD11b$^+$ cells from spleen. Initial gating of live cell population then on CD11b$^+$ population, as indicated. **B:** Increased CCR5 expression on CD11b$^+$ cells from blood ($P = 0.017$) and spleen ($P = 0.001$) tissues, but not retina ($P = 0.24$). **C:** Increased CCR2 expression only detected in spleen ($P = 0.0007$). **D:** Increased expression of CX3CR1 on CD11b$^+$ cells in retina and spleen, but not blood, of diabetic mice. Data are expressed as mean ± SD, and the unpaired Student's *t*-test is used to calculate the significance. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

the diabetic group (group 4) (45.1 ± 4.0 versus 48.6 ± 6.6; Figure 6). The effects of rosuvastatin were confirmed in whole mount preparations using adoptive transfer of calcein-AM–labeled cells (data not shown).

Discussion

Capillary occlusion, a hallmark sign of retinal ischemia in DR, occurs early in the disease and has been detected even before the onset of overt clinical funduscopic signs.[31] The role of intravascularly entrapped, adherent leukocytes (leukostasis) in mediating capillary occlusion is considered an important contributory factor, at least in experimental models of DR.[32–34] This has offered the possibility of novel therapeutic targets to interrupt the early development of DR. However, leukocytes are a diverse population of cells and have multiple functions, including release of inflammatory mediators, adhesion to endothelium, and migration into the tissues; the range of released growth factors, particularly VEGF, that attracts

Table 1. Increased Mean Fluorescence Intensity of CX3CR1 on CD11b$^+$ Cells of Diabetic Retinal Cell Suspension by Flow Cytometry

	CX3CR1 fluorescence		
Tissue	Healthy	Diabetic	*P* value
Blood	55.7 ± 42.9	61.5 ± 13.8	0.8346
Retina	28.6 ± 8.3	43.0 ± 9.1	0.0126*
Spleen	47.5 ± 3.1	18.5 ± 9.6	0.5233

Data are expressed as mean ± SD ($n = 7$), and the unpaired Student's *t* test was used to calculate the significance.
*$P < 0.05$.

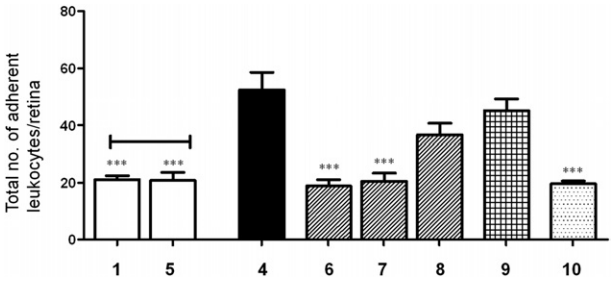

Figure 6. Total number of calcein-AM–labeled leukocytes adhering to the retinal vasculature *in vivo* in control and STZ-induced diabetic mice in a 30-minute observation period assessed by SLO: the effect of rosuvastatin treatment. Groups are defined as follows: 1, Cd→Cr; 2, Dd→Cr; 3, Cd→Dr; 4, Dd→Dr. C, control; D, diabetic; d, donor; r, recipient. Donor and recipient control mice are treated with pCd→pCr (group 5, *p* = rosuvastatin preventive treatment regimen) or without rosuvastatin (group 1). Donor and recipient diabetic mice are treated with or without rosuvastatin in a prevention protocol in various combinations (three groups with rosuvastatin, as follows: pDd→pDr, group 6; Dd→pDr, group 7; and pDd→Dr, group 8; and one group without rosuvastatin: Dd→Dr, group 4). One rosuvastatin group is also treated with mevalonate (pmDd→pmDr, group 9; m, mevalonate treatment). Finally, a further group is treated with rosuvastatin on an interventional protocol (see *Materials and Methods*) (iDd→iDr, group 10; i, rosuvastatin intervention treatment regimen). Significant differences between the groups are observed as indicated: **$P < 0.01$, ***$P < 0.001$ versus group 4.

them to tissues is equally diverse.[35] Accordingly, targeting leukocyte-endothelial cell interactions offers a dauntingly large range of options, and it would seem reasonable to focus efforts on specific aspects of the process.

This study was, therefore, designed to determine whether there was any selectivity in the type of leukocyte that is involved in leukostasis in the early stages of DR. The advantage of using the retinal whole mount technology is that the entire population of intraretinal leukocytes can be enumerated. The data show that $CD11b^+$ BM-derived myeloid cells were the predominant cell arresting within retinal capillaries. Although there is a small population of perivascular major histocompatibility complex class 11^+ cells in the healthy retina, the SLO experiments using calcein-AM–labeled cells (Figures 1 and 2), combined with the whole mounts (Figure 3), indicate that the $CD11b^+$ cells entrapped in the retinal vessels derive from the adoptively transferred circulating cells. The number of cells that became trapped per retina was relatively small (approximately 150 per retina) over the 1-hour time frame of the adoptive transfer study. However, this may account for a considerable effect if the half-life and the turnover of the monocyte are account for and the cell becomes fixedly adherent. Differences in transient adherence versus prolonged adhesion within retinal capillaries were shown in the *in vivo* SLO experiments (Figure 1), whereas the whole mount experiments indicated that long-standing, if not permanent, adhesion of leukocytes also occurred. How this converts into actual areas of retinal ischemia will depend on many factors, including the ability of the retinal vasculature to repair itself, by either opening new capillary channels or recanalizing older acellular capillaries. Thus, a significant period may elapse between the onset of diabetes, the development of leukostasis-mediated capillary occlusion, and the appearance of clinically overt signs of retinopathy, as occur in human DR. In addition, the ischemic stimulus and the release of VEGF may have to reach a threshold level before frank neovascularization occurs, although capillary leakage could occur at an earlier stage.

Targeting the earliest stages of the disease may, therefore, be a useful therapeutic strategy. In this context, it is important to know which stimuli are important for leukocyte homing to the retina. Chemokines, presumably released from the tissues into the bloodstream, are believed to be important in directing leukocytes to sites of inflammation, and do so by ligating specific receptors on the leukocyte surface.[36] Interestingly, as previously indicated, polymorphisms in the CCR5 receptor have been associated with diabetes. In this study, we have observed an increase in numbers of $CCR5^+$ cells within the $CD11b^+$ population of presumed monocytes/macrophages in the blood and the spleen in diabetic mice compared with healthy mice (Figure 5B). This suggests that CCR5 may be an important receptor in guiding circulating mononuclear cells, activated systemically in diabetes, to sites of *para*-inflammation, such as the diabetic retina.[18] Interestingly, the expression of CCR5 on $CD11b^+$ cells was not different between intravascular trapped leukocytes in healthy retina compared with diabetic retina, suggesting that CCR5 expression may be reduced once the cells become firmly adherent to the vessel wall. Alternatively, it is possible that $CCR5^+$ is normally required for entrapment of cells in the capillary vasculature, and that its up-regulation in diabetes increases this risk.

Also of interest was the observation that the CCR2 receptor, normally associated with migration of inflammatory monocytes to sites of acute inflammation,[37] was not increased on circulating $CD11b^+$ leukocytes in diabetic mice nor on the cells entrapped in the retina (Figure 5C). This likely supports the notion that inflammation associated with high plasma glucose levels in DR is low grade and chronic.[18] In contrast, we also observed that diabetic $CD11b^+$ leukocytes preferentially expressed the CX3CR1 receptor in the accumulated cells in both the retina and spleen (Figure 5D). CX3CR1 expression is associated with a subset of $Lys6C^{lo}$ monocytes, which tend to populate the tissues as resident macrophages.[38] However, both $CCR2^+$ and $CX3CR1^+$ monocytes, expressing high levels of CCR5, appear to be required for the pathogenesis of atherosclerotic disease,[38] a major associated comorbidity of diabetes. Our data suggest, therefore, that there exists a complex cross talk between different monocytic subsets in the development of the complications of diabetes, such as retinopathy, and in non–diabetes-associated atherosclerosis.[39] Of further relevance to the pathogenesis of DR, it has recently been shown that fractalkine, the CX3CR1 ligand, has angiogenic properties and is elevated in vitreous samples from patients with proliferative DR.[40] These data implicate the leukocyte as the primary mediator of leukocyte-endothelial adhesion. However, despite these clear changes in chemokine expression in diabetic leukocytes, the data obtained with rosuvastatin treatment indicate that the major metabolic defect corrected by rosuvastatin lies with the endothelial cells and that diabetes-induced changes in the endothelium were a prerequisite for the development of leukostasis. It is possible, therefore, that the changes in leukocyte phenotype and function are secondary to changes in the endothelium, but recent evidence suggests that changes in BM cells in diabetes are the result of diabetes-associated neuropathic damage.[41] This has also been attributed to primary dysfunction in the endothelium of the small vessels.[42,43]

In conclusion, this study has confirmed the role of leukocytes in causing capillary leukostasis in early DR, has identified the $CD11b^+$ monocyte/macrophage as the central cell mediating this pathological characteristic, and has indicated that this cell is activated in diabetes by up-regulating the expression of chemokines, including CCR5 and CCR2. However, the main site of metabolic dysfunction at which rosuvastatin regulates leukocyte-endothelial cell adhesion appears to be located at the endothelial cell and involves the HMG-CoA reductase pathway.

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References

1. Chew EY, Ambrosius WT, Davis MD, Danis RP, Gangaputra S, Greven CM, Hubbard L, Esser BA, Lovato JF, Perdue LH, Goff DC Jr, Cushman WC, Ginsberg HN, Elam MB, Genuth S, Gerstein HC, Schubart U, Fine LJ, Grp AS, Grp AES: Effects of medical therapies on retinopathy progression in type 2 diabetes. N Engl J Med 2010, 363:233–244
2. Borsey DQ, Prowse CV, Gray RS, Dawes J, James K, Elton RA, Clarke BF: Platelet and coagulation-factors in proliferative diabetic-retinopathy. J Clin Pathol 1984, 37:659–664
3. Maiello M, Boeri D, Bonadonna R, Odetti P, Saccarello A, Adezati L: Platelet and clotting activities after cold stress in diabetic-patients. Thromb Res 1988, 50:885–894
4. Vicari AM, Macagni A: Primary platelet activation in recent-onset type-1 diabetes-mellitus. Scand J Clin Lab Invest 1990, 50:429–432
5. Adamis AP: Is diabetic retinopathy an inflammatory disease? Br J Ophthalmol 2002, 86:363–365
6. Joussen AM, Poulaki V, Le ML, Koizumi K, Esser C, Janicki H, Schraermeyer U, Kociok N, Fauser S, Kirchhof B, Kern TS, Adamis AP: A central role for inflammation in the pathogenesis of diabetic retinopathy. FASEB J 2004, 18:1450–1452
7. Miyamoto K, Hiroshiba N, Tsujikawa A, Ogura Y: In vivo demonstration of increased leukocyte entrapment in retinal microcirculation of diabetic rats. Invest Ophthalmol Vis Sci 1998, 39:2190–2194
8. Schroder S, Palinski W, Schmidschonbein GW: Activated monocytes and granulocytes, capillary nonperfusion, and neovascularization in diabetic-retinopathy. Am J Pathol 1991, 139:81–100
9. Navarro-Gonzalez JF, Mora-Fernandez C, Muros de Fuentes M, Garcia-Perez J: Inflammatory molecules and pathways in the pathogenesis of diabetic nephropathy. Nat Rev Nephrol 2011, 7:327–340
10. Muntinghe FL, Gross S, Bakker SJ, Landman GW, van der Harst P, Bilo HJ, Navis G, Zuurman MW: CCR5Δ32 genotype is associated with outcome in type 2 diabetes mellitus. Diabetes Res Clin Pract 2009, 86:140–145
11. Kim SY, Johnson MA, McLeod DS, Alexander T, Hansen BC, Lutty GA: Neutrophils are associated with capillary closure in spontaneously diabetic monkey retinas. Diabetes 2005, 54:1534–1542
12. Barouch FC, Miyamoto K, Allport JR, Fujita K, Bursell SE, Aiello LP, Luscinskas FW, Adamis AP: Integrin-mediated neutrophil adhesion and retinal leukostasis in diabetes. Invest Ophthalmol Vis Sci 2000, 41:1153–1158
13. Bai N, Tang S, Ma J, Luo Y, Lin S: Increased expression of intercellular adhesion molecule-1, vascular cellular adhesion molecule-1 and leukocyte common antigen in diabetic rat retina. Yan Ke Xue Bao 2003, 19:176–183
14. Limb GA, Webster L, Soomro H, Janikoun S, Shilling J: Platelet expression of tumour necrosis factor-alpha (TNF-alpha): TNF receptors and intercellular adhesion molecule-1 (ICAM-1) in patients with proliferative diabetic retinopathy. Clin Exp Immunol 1999, 118:213–218
15. 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
16. Miyamoto K, Ogura Y: Pathogenetic potential of leukocytes in diabetic retinopathy. Semin Ophthalmol 1999, 14:233–239
17. Stalker TJ, Skvarka CB, Scalia R: A novel role for calpains in the endothelial dysfunction of hyperglycemia. FASEB J 2003, 17:1511–1513
18. Leal EC, Aveleira CA, Castilho AF, Serra AM, Baptista FI, Hosoya K, Forrester JV, Ambrósio AF: High glucose and oxidative/nitrosative stress conditions induce apoptosis in retinal endothelial cells by a caspase-independent pathway. Exp Eye Res 2009, 88:983–991
19. Ishida S, Usui T, Yamashiro K, Kaji Y, Amano S, Ogura Y, Hida T, Oguchi Y, Ambati J, Miller JW, Gragoudas ES, Ng YS, D'Amore PA, Shima DT, Adamis AP: VEGF164-mediated inflammation is required for pathological, but not physiological, ischemia-induced retinal neovascularisation. J Exp Med 2003, 198:483–489
20. Wang J, Xu X, Elliott MH, Zhu M, Le Y-Z: Muller cell-derived VEGF is essential for diabetes-induced retinal inflammation and vascular leakage. Diabetes 2010, 59:2297–2305
21. Hirata F, Yoshida M, Ogura Y: High glucose exacerbates neutrophil adhesion to human retinal endothelial cells. Exp Eye Res 2006, 82:179–182
22. Chavakis T, Bierhaus A, Al-Fakhri N, Schneider D, Witte S, Linn T, Nagashima M, Morser J, Arnold B, Preissner KT, Nawroth PP: The pattern recognition receptor (RAGE) is a counterreceptor for leukocyte integrins: a novel pathway for inflammatory cell recruitment. J Exp Med 2003, 198:1507–1515
23. Olson JA, Whitelaw CM, McHardy KC, Pearson DWM, Forrester JV: Soluble leucocyte adhesion molecules in diabetic retinopathy stimulate retinal capillary endothelial cell migration. Diabetologia 1997, 40:1166–1171
24. MacKinnon JR, Knott RM, Forrester JV: Altered L-selectin expression in lymphocytes and increased adhesion to endothelium in patients with diabetic retinopathy. Br J Ophthalmol 2004, 88:1137–1141
25. Xu H, Dawson R, Forrester JV, Liversidge J: Identification of novel dendritic cell populations in normal mouse retina. Invest Ophthalmol Vis Sci 2007, 48:1701–1710
26. Manivannan A, Sharp PF, Phillips RP, Forrester JV: Digital fundus imaging using a scanning laser ophthalmoscope. Physiol Meas 1993, 14:43–56
27. Xu HP, Manivannan A, Goatman KA, Liversidge J, Sharp PF, Forrester JV, Crane IJ: Improved leukocyte tracking in mouse retinal and choroidal circulation. Exp Eye Res 2002, 74:403–410
28. Leal EC, Manivannan A, Hosoya K-I, Terasaki T, Cunha-Vaz J, Ambrosio AF, Forrester JV: Inducible nitric oxide synthase isoform is a key mediator of leukostasis and blood-retinal barrier breakdown in diabetic retinopathy. Invest Ophthalmol Vis Sci 2007, 48:5257–5265
29. Mooyaart AL, Valk EJJ, van Es LA, Bruijn JA, de Heer E, Freedman BI, Dekkers OM, Baelde HJ: Genetic associations in diabetic nephropathy: a meta-analysis. Diabetologia 2011, 54:544–553
30. Solomon M, Balasa B, Sarvetnick N: CCR2 and CCR5 chemokine receptors differentially influence the development of autoimmune diabetes in the NOD mouse. Autoimmunity 2010, 43:156–163
31. Tam J, Dhamdhere KP, Tiruveedhula P, Manzanera S, Barez S, Bearse MA Jr, Adams AJ, Roorda A: Disruption of the retinal parafoveal capillary network in type 2 diabetes before the onset of diabetic retinopathy. Invest Ophthalmol Vis Sci 2011, 52:9257–9266
32. Adamis AP, Berman AJ: Immunological mechanisms in the pathogenesis of diahetic retinopathy. Semin Immunopathol 2008, 30:65–84
33. Kern TS: Contributions of inflammatory processes to the development of the early stages of diabetic retinopathy. Exp Diabetes Res 2007, 2007:95103
34. Chibber R, Ben-Mahmud BM, Chibber S, Kohner EM: Leukocytes in diabetic retinopathy. Curr Diabetes Rev 2007, 3:3–14
35. Sharma M: Chemokines and their receptors: orchestrating a fine balance between health and disease. Crit Rev Biotechnol 2010, 30:1–22
36. Langer HF, Chavakis T: Leukocyte-endothelial interactions in inflammation. J Cell Mol Med 2009, 13:1211–1220
37. Geissmann F, Jung S, Littman DR: Blood monocytes consist of two principal subsets with distinct migratory properties. Immunity 2003, 19:71–82
38. Gautier EL, Jakubzick C, Randolph GJ: Regulation of the migration and survival of monocyte subsets by chemokine receptors and its relevance to atherosclerosis. Arterioscler Thromb Vasc Biol 2009, 29:1412–1418
39. Gautier EL, Huby T, Saint-Charles F, Ouzilleau B, Pirault J, Deswaerte V, Ginhoux F, Miller ER, Witztum JL, Chapman MJ, Lesnik P: Conventional dendritic cells at the crossroads between immunity and cholesterol homeostasis in atherosclerosis. Circulation 2009, 119:2367–2375
40. You J-J, Yang C-H, Huang J-S, Chen M-S, Yang C-M: Fractalkine, a CX3C chemokine, as a mediator of ocular angiogenesis. Invest Ophthalmol Vis Sci 2007, 48:5290–5298
41. Busik JV, Tikhonenko M, Bhatwadekar A, Opreanu M, Yakubova N, Caballero S, Player D, Nakagawa T, Afzal A, Kielczewski J, Sochacki A, Hasty S, Calzi SL, Kim S, Duclas SK, Segal MS, Guberski DL, Esselman WJ, Boulton ME, Grant MB: Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. J Exp Med 2009, 206:2897–2906
42. Plutzky J: Macrovascular effects and safety issues of therapies for type 2 diabetes. Am J Cardiol 2011, 108:25B–32B
43. Cameron NE, Eaton SEM, Cotter MA, Tesfaye S: Vascular factors and metabolic interactions in the pathogenesis of diabetic neuropathy. Diabetologia 2001, 44:1973–1988