Circulating Mononuclear Progenitor Cells: Differential Roles for Subpopulations in Repair of Retinal Vascular Injury

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Purpose. We examined effect on retinal vascular homing of exogenous $CD34^+$ and $CD14^+$ progenitor cells using mouse models of chronic (streptozotocin [STZ]-induced diabetes) and acute (ischemia–reperfusion [I/R]) ocular vascular injury.

Methods. STZ-treated mice of short or long duration ($\leq$4, $\geq$11 months) diabetes, along with age- and sex-matched controls, were given intravitreous injections of human $CD34^+$ and $CD14^+$ cells isolated from healthy or diabetic donors alone or in combination. I/R injured mice were given diabetic or nondiabetic $CD34^+$ cells with mesenchymal stem cells (MSCs) or diabetic $CD34^+$ cells manipulated by ex vivo fucosylation with ASC-101. Injected cells were localized by fluorescent immunocytochemistry, and the degree of retinal vascular colocalization quantified morphometrically. Permeability was assessed by fluorescent albumin leakage.

Results. Diabetic $CD14^+$ cells associated with vessels to a greater degree than diabetic $CD34^+$ cells. Vascular permeability was reduced only by nondiabetic cells and only at the highest number of cells tested. Diabetic $CD34^+$ cells consistently demonstrated reduced migration. There was a 2-fold or 4-fold increase over control in the specific localization of diabetic $CD34^+$ cells within the vasculature when these cells were co-administered with MSCs or ex vivo fucosylated prior to injection, respectively.

Conclusions. Diabetic $CD14^+$ cells, unlike diabetic $CD34^+$ cells, retain robust homing characteristics. $CD34^+$ or $CD14^+$ subsets rather than whole bone marrow or peripheral blood cells may prove more beneficial in autologous cell therapy for diabetics. Co-administration with MSCs or ex vivo fucosylation may enhance utility of $CD34^+$ cells in cell therapy for diabetic ocular conditions like macular ischemia and retinal nonperfusion.

Keywords: progenitor cells, neovascularization, diabetic retinopathy

Retinal vascular diseases, such as diabetic retinopathy, remain a common cause of vision loss and blindness. Diabetes can damage the small blood vessels, leading to retinal nonperfusion and ischemia. Although therapies are available to treat aspects of diabetic retinopathy, no therapy is available to correct damaged retinal vasculature and eliminate ischemia. For example, macular ischemia is vision threatening and currently has no treatment.

Bone marrow–derived stem cells are the major source of hematopoietic precursors and vascular progenitors.[1–4] Research over the last decade has shown that bone marrow–derived progenitor cells are capable of homing to vascular lesions in the eye and contribute to physiological vascular repair.[3,5–8] These cells have been implicated in the formation of normal, functioning ocular vasculature during development of the newborn eye and in restoration of retinal function when injected into eyes of animal models with retinal degeneration, thus supporting the therapeutic potential of these cells.[9–11] However, the degree of progenitor cell involvement in vascular repair appears to vary with vascular bed, local growth stimuli, and disease state (e.g., diabetes).[3,4,12–14]

Bone marrow–derived cells that express the inflammatory monocyte marker CD14 are known to exhibit endothelial characteristics under certain conditions.[4,15–17] These cells have been shown to effect vascular repair in ischemic hind limb models of vascular injury.[18] Umbilical cord–derived $CD14^+$ monocytes can correct ischemia[19] and show beneficial effect in a stroke model.[20] $CD14^+$ cells can differentiate into endothelial cells[21] as well as neuronal[22] and mature myeloid cells. The tissue microenvironment to which monocytes are exposed influences their fate into either pro- or anti-inflammatory macrophages (M1 and M2, respectively),[23] and determines whether potentially beneficial or deleterious effects occur during vascular remodeling. $CD14^+$ cells polarized to M2 macrophages following injection into the eyes of mice undergoing oxygen-induced retinopathy and exerted a profound rescue effect to reduce pathological neovascularization.[24] Monocytes can also act as "bridge cells" when recruited from the circulation to establish tip-cell anastomosis and release

of angiogenic factors to promote vessel fusion and vascular network formation.[25]

Preclinical studies have investigated the use of bone marrow stem/progenitor cells to treat diabetes mellitus, genetic kidney disease, strokes, spinal cord injury, and hepatic cirrhosis.[26–28] Bone marrow cells, either unfractionated or fractionated, have been investigated in clinical trials as therapy for various ischemic or degenerative conditions. Infusion of autologous bone marrow stem/progenitor cells into the coronary artery is being used therapeutically in patients with recent myocardial infarct to minimize cardiac failure.[29,30]

Injecting bone marrow stem cells directly into the eye to treat retinal disease has been explored using animal models.[9,12,31–33] An intravitreous route of administration of cells is appealing since the procedure is safe, inexpensive, and easily accomplished. Fewer cells are needed, and these cells may stay in the eye for an extended period and potentially have a longer lasting effect than if they were injected into the systemic circulation. $CD34^+$ bone marrow cells have been given by intravitreous injection in murine eyes after mechanical injury of the retina and have been shown to incorporate into the outer retina in the area of injury for up to a year.[34]

Therefore, the use of bone marrow stem cells, specifically human $CD34^+$ cells, is an exciting potential therapy for the treatment of retinal vascular disease in humans. Ideally, patients would be treated with their own cells, and this would eliminate any complications resulting from rejection of donor stem/progenitor cells. Complicating the issue, however, is the observation that $CD34^+$ cells from diabetic patients are dysfunctional. They do not migrate to the sites of ischemic damage or proliferate like normal human $CD34^+$ cells. Therefore, their use is limited. Previously, we demonstrated that circulating $CD34^+$ cells from human diabetic patients could neither repair acellular capillaries in the retina of a type 2 diabetes model nor repair retina of a streptozotocin (STZ)-induced diabetic model. In contrast, $CD34^+$ cells from nondiabetic individuals were able to do so.[12]

In an attempt to better understand the cell populations that can be used for autologous cell therapy, we conducted a series of studies using human progenitor populations in a type 1 diabetes model and in a model of acellular capillaries that recapitulates many of the histological features of diabetic retinopathy, the ischemia–reperfusion (I/R) injury model. We tested two combinations of cells: $CD34^+$ cells with either autologous $CD14^+$ or bone marrow–derived mesenchymal stem cells (MSCs). Finally, we evaluated a novel technology of ex vivo modification of diabetic $CD34^+$ cells for its efficacy in facilitating the targeting of $CD34^+$ cells to areas of retinal injury.

Methods

Animals

All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Florida and at Case Western Reserve University, and studies were conducted in accordance with the principles described in the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Normal female C57Bl/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME) and were made diabetic by injection of STZ as previously described.[35] Duration of diabetes was specific to each experiment as further described later. Animals were made diabetic at 2 months of age and were maintained in a diabetic state for either 4 months (chronological age of 6 months) or at least 11 months (chronological age 13 months). An additional cohort of animals of equivalent ages was maintained for use as nondiabetic age-matched controls. Exact numbers of animals used is detailed for each experiment, described in following text. Selected studies in nondiabetic animals used a short-term injury model of ocular ischemia and reperfusion as previously described.[12]

At study termination the animals were euthanized by overdose of ketamine and xylazine (14 and 30 mg/kg, respectively) followed by thoracotomy, at which time the eyes were removed for immunohistochemical processing.

Human $CD34^+$ and $CD14^+$ Peripheral Blood Mononuclear Cells

Human cells were obtained from blood samples of individuals seen at University of Florida clinics after they gave informed consent and in accordance with the approved protocol by the Institutional Review Board. The inclusion criterion for the study was diabetes for at least 10 years. Control patients (without diabetes) were matched for age and sex of diabetic individuals.

Blood was collected from donors using cell preparation tube with sodium heparin sodium as the anticoagulant. After density gradient centrifugation, the buffy coat containing leukocytes was collected. Red blood cell contamination was removed using ammonium chloride solution (Stem Cell Technologies, Vancouver, BC). The resulting mononuclear cells were enriched for $CD34^+$ cells by positive selection using a human CD34 selection kit (Stem Cell Technologies), while the $CD14^+$ cells were enriched from the $CD34^-$ fraction by a human monocyte enrichment kit (Stem Cell Technologies) according to the manufacturer's instructions. The enriched $CD34^+$ and $CD14^+$ cells that were administered to STZ-diabetic animals were kept on ice in media (Stem Span Media; Stem Cell Technologies) and injected within 24 hours of isolation. Cells used in the I/R injury model animals were injected within 1 hour of isolation and were not exposed to media.

Mesenchymal Stem Cells

Murine MSCs were isolated from bone marrow and enriched by fluorescence activated cell sorting as previously described.[36]

$CD34^+$ Cells in Young Versus Aged Mice

Our first studies examined the effect of age and duration of diabetes on $CD34^+$ cell function in vascular maintenance and repair. For this study mice with STZ diabetes duration of 4 ± 0.5 months ($n = 8$) and $\geq$11 months ($n = 8$), along with age-matched normal controls ($n = 8$ for each of the duration points) were used. Animals in either the STZ-diabetic 4-month group, STZ-diabetic 11-month group, or the corresponding age-matched control groups were administered human $CD34^+$ cells from either nondiabetic or diabetic donors by intravitreous injection at a dose of 10×10^3 cells in 1 μL.

$CD14^+$ Cells in Aged Mice

Mice with STZ diabetes of $\geq$11 months' duration ($n = 6$), along with age-matched normal controls ($n = 6$) were used. Animals were then randomly divided into groups and given human $CD14^+$ endothelial progenitor cells (EPCs) from either nondiabetic or diabetic donors by intravitreous injection at a dose of 10×10^3 cells in 1 μL.

Co-injection of $CD34^+$ and $CD14^+$ Cells in Aged Mice

Mice with STZ diabetes of $\geq$11 months' duration ($n = 6$) were given an intravitreous injection (20×10^3 cells in 1 μL) of a 1:1

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mixture of $CD34^+$ and $CD14^+$ cells. Three of the mice received cells from one patient, while the remaining three mice received cells from a second patient. Prior to injection, cells were fluorescently labeled using nanoparticles (Qtracker 525 or Qtracker 625; Invitrogen, Eugene, OR) according to the manufacturer's protocols. Cells were washed by centrifugation three times in PBS before adjusting final concentration for injection.

Co-injection of $CD34^+$ Cells With MSCs

Mice subjected to the I/R injury model ($n = 6$) received intravitreous injection of a 1:1 mix of diabetic $CD34^+$ peripheral blood EPCs and mouse MSCs at a dose of 20×10^3 cells in 1 μL. Additional age- and sex-matched mice with I/R injury ($n = 6$) were given diabetic $CD34^+$ EPCs alone (10×10^3 cells/μL/eye). Eyes were then harvested 2 days after injection.

Effect of Peripheral Blood EPC Concentration on Vascular Association

Mice subjected to the I/R injury model received intravitreous injection of one of the following 12 dosings of cells ($n = 5$ for each condition): 1×10^3 $CD34^+$ cells from a normal donor; 1×10^3 $CD14^+$ cells from the same normal donor; 1×10^3 $CD34^+$ cells plus 1×10^3 $CD14^+$ cells from the same normal donor; 5×10^3 $CD34^+$ cells from a normal donor; 5×10^3 $CD14^+$ cells from the same normal donor; 5×10^3 $CD34^+$ cells plus 5×10^3 $CD14^+$ cells from the same normal donor; 1×10^3 $CD34^+$ cells from a diabetic donor; 1×10^3 $CD14^+$ cells from the same diabetic donor; 1×10^3 $CD34^+$ cells plus 1×10^3 $CD14^+$ cells from the same diabetic donor; 5×10^3 $CD34^+$ cells from a diabetic donor; 5×10^3 $CD14^+$ cells from the same diabetic donor; or 5×10^3 $CD34^+$ cells plus 5×10^3 $CD14^+$ cells from the same diabetic donor. Prior to injection, cells were fluorescently labeled using nanoparticles (Qtracker 525 or Qtracker 625; Invitrogen) according to manufacturer's protocols. Cells were washed by centrifugation three times in PBS before adjusting final concentration for injection. Eyes were harvested 2 days after injection.

Assessment of Changes in Vascular Permeability With the Addition Peripheral Blood Diabetic EPCs

Mice subjected to the I/R injury model ($n = 3$ for each condition) received intravitreous injection of the identical dosings of cells described in the previous section. An additional cohort of mice ($n = 3$) were given 10×10^3 mouse MSCs alone. Uninjured eyes ($n = 3$) and injured eyes that did not receive intravitreous injection of cells ($n = 3$) were used as negative and positive controls, respectively. Vascular permeability was measured 2 days after injection of cells as we have previously described.[36]

Ex Vivo Modification of Diabetic $CD34^+$ Cells

Isolated $huCD34^+$ cells from diabetic patients were treated using a proprietary technology, ASC-101, which contains fucosyltransferase VI and the substrate GDP-fucose. The subsequent enzymatically mediated, site- and stereospecific addition of fucose to sLex components that adhere to cell surface glycoproteins (fucosylation) leads to an increase in selectin-mediated binding between the treated stem cells and the endothelial luminal surface. Fucosylated $CD34^+$ cells from diabetic donors or untreated cells were then injected into the vitreous cavity of mouse eyes ($n = 8$ for each condition) that had been subjected to I/R injury 7 days prior. Eyes were harvested 2 days after injection of the cells.

Tissue Preparation and Immunocytochemistry

Enucleated eyes were pierced 1 mm posterior to the limbus with a 27-gauge needle and immersed in freshly prepared buffered 4% (w/v) paraformaldehyde for 1 hour at room temperature, then washed by immersion in two changes of PBS before dissection. Neural retina was isolated intact and permeabilized overnight at 4°C in HEPES-buffered saline containing 0.1% (v/v) Tween 20 and 1% (w/v) bovine serum albumin, and 10% nonimmune goat serum (Sigma-Aldrich, St. Louis, MO) with 10 μg/mL rhodamine-conjugated *Ricinus communis* agglutinin I (Vector Laboratories, Burlingame, VT). In experiments where the $CD34^+$ and $CD14^+$ cells were co-injected, the retinas were reacted with rabbit anticollagen IV (Abcam, San Francisco, CA) followed by goat antirabbit IgG conjugate to DyLight 649 (Abcam) to label vasculature, instead of agglutinin I. Human peripheral blood monocytes that were not labeled prior to injection were subsequently labeled for visualization in the retinas by further reacting the tissue with mouse antihuman histone H1 (Abcam) followed by goat antimouse IgG conjugated to DyLight 488 (Abcam) to label the human cells.

Data Collection and Analysis

Retinas were mounted flat by four to six radial incisions and placed between glass coverslips with antifade medium (Vectashield; Vector Laboratories). Digital image captures were made using either a spinning disc confocal microscope (Olympus DSU-IX81; Olympus Corporation of America, Center Valley, PA) or a laser scanning confocal microscope (Leica TCS SP2; Leica Microsystems, Buffalo Grove, IL). Z-series captures (3 μm z-depth) were made through the entire retinal thickness, yielding approximately 30 to 35 images per stack. A minimum of three random fields was captured for each retina.

Software-assisted color thresholding and density slicing was used to measure the area of fluorescence for each channel used in the spatially calibrated images (ImageJ; NIH Research Service Branch, http://rsb.info.nih.gov/ij/index.html). Colocalization of markers (where appropriate) was accomplished in software prior to morphometric analysis (Intensity Correlation Analysis plugin; MBF Plugin Collection, http://rsbweb.nih.gov/ij/plugins/mbf-collection.html) and was calculated as a function of total vascular area within the field.[6]

Values for area were summed for the entire stack. Summed areas for each stack were then averaged for each condition. Values for each stack were then averaged for a single eye, thus providing a single value for each animal. Those values were then used to determine differences between the various groups by *F*-test for the null hypothesis, with known variance. A $P \leq 0.05$ was considered significant.

Results

$CD34^+$ Cells in Young Versus Aged Mice

Greater colocalization of healthy $huCD34^+$ cells with retinal vessels was observed in aged animals (13 months, age-matched control for 11 months of STZ-induced diabetes) versus young animals (6 months, age-matched control for 4 months of STZ-induced diabetes; Fig. 1A versus Figs. 1C, 1I [white bars], $P < 0.05$). This colocalization of cells within aged vessels was considerably less than that seen in diabetes, as evidenced by the increased colocalization of healthy donor $CD34^+$ cells with vasculature in diabetic recipients (Fig. 1E versus Figs. 1G, 1I [black bars], $P < 0.05$ versus normal age-matched recipient).

When cells from diabetic donors were used, they showed a reduced colocalization with vasculature from nondiabetic aged

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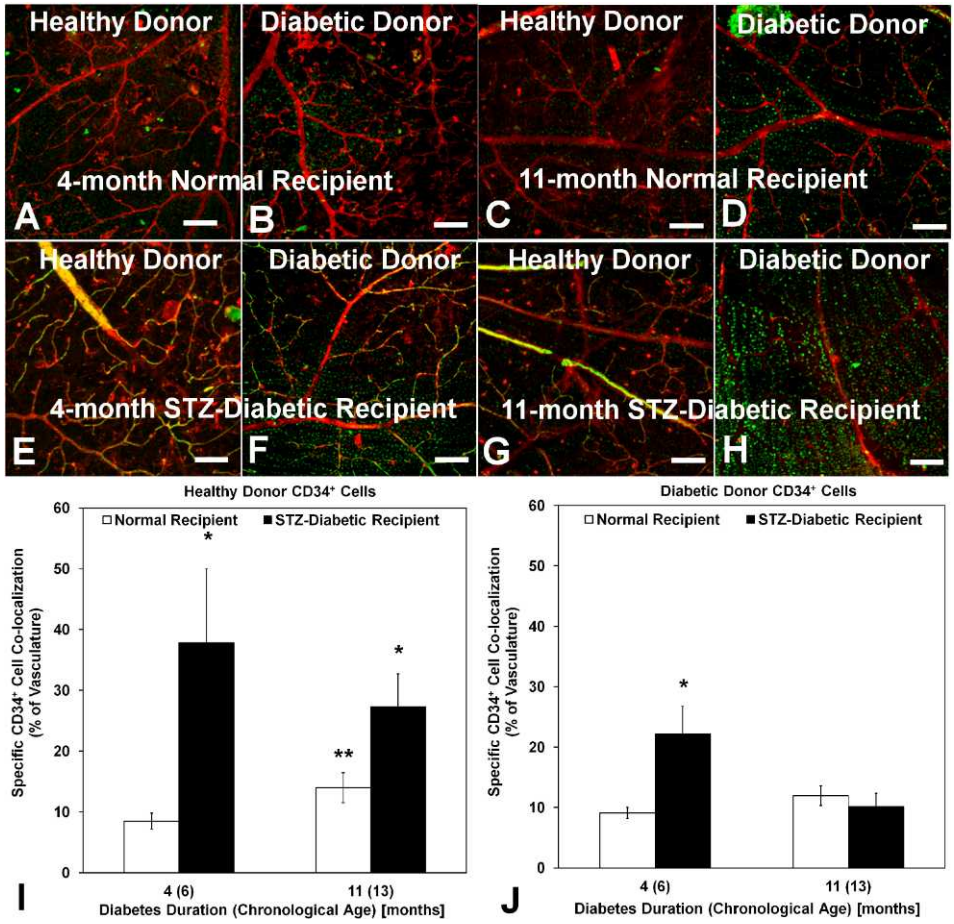

Figure 1. Age increases vascular damage, although not to the degree of long-term diabetes. Representative and typical confocal microscope image captures showing retinal vascular colocalization of CD34$^+$ cells from either healthy (**A**, **C**, **E**, **G**) or diabetic (**B**, **D**, **F**, **H**) donors given by intravitreous injection to healthy age-matched control mice (**A**–**D**) and their STZ-diabetic counterparts (**E**–**H**). Note the extensive colocalization between the *red*-labeled vasculature (rhodamine-conjugated *R. communis* agglutinin I) and the *green*-labeled cells (mouse antihuman histone H1 followed by goat antimouse IgG conjugated to DyLight 488) in (**B**) and (**D**), in which a mouse received healthy CD34$^+$ cells after either 4 or 11 months of STZ-induced diabetes, respectively, and contrast with (**F**) and (**H**), in which diabetic donor cells were used. *Scale bar* = 50 μm. (**I**) and (**J**) summarize the quantitative results of measuring specific retinal vascular colocalization of the injected CD34$^+$ cells from healthy or diabetic donors, respectively, in the groups with two different durations of diabetes. Each *bar* represents $n = 8$ animals; $^{**}P < 0.05$ compared to 4-month normal recipient; $^{*}P < 0.05$ compared to respective age-matched normal recipient.

animals (Figs. 1D, 1J), compared to cells from healthy donors. Diabetic donor cells did, however, demonstrate increased colocalization to vasculature in short-term (4 months) STZ-diabetic recipients (Fig. 1F), although considerably less than the colocalization shown by healthy donor cells. By 11 months of diabetes in the recipient, the diabetic donor cells showed little or no vascular association (Fig. 1H) as we previously reported.[12]

CD14$^+$ Cells in Aged Mice

Circulating CD14$^+$ monocytes isolated from healthy donors colocalized with retinal vessels (mostly capillaries) of aged nondiabetic recipient mice (Fig. 2A) to approximately the same degree as did nondiabetic CD34$^+$ cells in aged nondiabetic mice, encompassing approximately 15% of vascular area (Fig. 2F versus Fig. 1I). On the other hand, CD14$^+$ cells from diabetic donors exhibited a greater degree of vessel colocalization in aged nondiabetic mice (Fig. 2B) than did diabetic CD34$^+$ cells, encompassing 21 ± 1.5% vs. 12 ± 2.5% of vascular area, respectively ($P < 0.05$). As with nondiabetic CD14$^+$ cells, the diabetic CD14$^+$ cells associated primarily with smaller vessels.

When the recipient animals were aged and diabetic, CD14$^+$ cells from healthy donors colocalized to vasculature (Fig. 2C) to a slightly, but not significantly, lesser degree than did diabetic CD34$^+$ cells (22 ± 1.6% vs. 27 ± 5.4% of vasculature). Curiously, CD14$^+$ cells from diabetic donors colocalized with retinal vessels in normal mice (Fig. 2B) to the same degree as did healthy CD34$^+$ cells in STZ-diabetic mice. In these diabetic animals, the cells colocalized with both small and midsized (arteriole/venule) vessels.

CD14$^+$ cells from diabetic donors showed significantly more vessel colocalization in aged diabetic animals (Fig. 2D) than did diabetic CD34$^+$ cells (Fig. 1H), comprising up to 27 ± 2.0% vs. 11 ± 2.3% of vasculature, respectively ($P < 0.05$), approximately to the same degree as did healthy CD34$^+$ cells in STZ-diabetic recipients. Diabetic CD14$^+$ cells also colocalized with retinal vessels in diabetic animals to a significantly greater degree than did CD14$^+$ cells from healthy donors ($P < 0.05$).

The distribution of these cells through the thickness of the vascular plexus is depicted in Figure 2E, which shows an orthogonal three-dimensional projection along the plane of the *x*-axis from a z-series of 35 captures through the entire thickness of the vessel compartment. The green fluorescence-

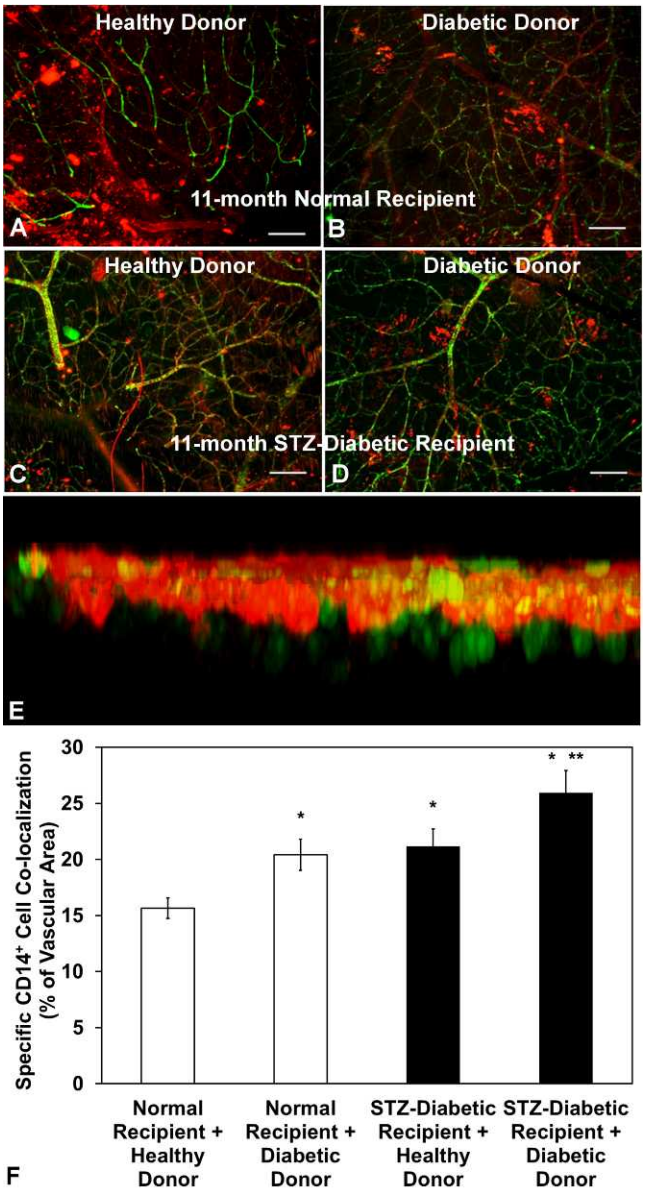

Figure 2. Diabetic $CD14^+$ cells colocalize to retinal vasculature damaged by chronic diabetes more than healthy $CD14^+$ cells do. Representative confocal microscope image captures (compressed z-series) show that human $CD14^+$ cells from healthy donors demonstrate a large degree of colocalization to damaged retinal vasculature, whether the damage occurs from age alone (**A**) or from long-term STZ-induced diabetes (**C**), while $CD14^+$ cells from diabetic donors colocalize with damaged retinal vessels to a greater degree (**B**, **D**). Unlike diabetic $CD34^+$ cells, diabetic $CD14^+$ cells colocalize extensively with diabetic vessels, both venule/arteriole and capillaries. *Scale bar* = 50 μm. A three-dimensional projection of a retina from an 11-month STZ-diabetic mouse that received $CD14^+$ cells from a diabetic donor is shown in cross-section in (**E**). Note that the *green*-labeled CD14 cells are seen in both perivascular and integral loci, with the majority of perivascular cells on the anterior portion of the neural retinal vascular plexus (*bottom* of the frame as depicted). Quantitative measurement of specific colocalization as a function of vascular area is shown in (**F**). Each *bar* represents $n = 6$ animals; $^*P < 0.05$ compared to age-matched normal recipient given healthy donor $CD14^+$ cells. $^{**}P < 0.05$ compared to age-matched diabetic recipient given healthy donor $CD14^+$ cells. Vasculature was labeled with rhodamine-conjugated *R. communis* agglutinin I, while cells of human origin (the injected $CD14^+$ cells) were labeled with mouse antihuman histone H1 followed by goat antimouse IgG conjugated to DyLight 488 as described in the Methods.

labeled $CD14^+$ cells are seen both perivascularly as well as deep with the red-fluorescence-labeled vessel plane, with most of the perivascular cells occurring on the anterior side of the plexus (bottom of the frame as depicted).

Co-injection of $CD34^+$ and $CD14^+$ Cells in Aged Mice

When diabetic $CD34^+$ and $CD14^+$ cells were injected in a 1:1 mixture into eyes of aged diabetic mice, the degree of $CD34^+$ cell colocalization (Figs. 3B, 3D) was the same as when $CD34^+$ cells were administered alone (approximately 12% in both cases, compare to Fig. 1J). However, $CD14^+$ colocalization (Figs. 3C, 3E) was diminished compared to $CD14^+$ cells given alone (Fig. 2E), from 27 ± 2.0% to 17 ± 1.7% of vasculature, respectively (Fig. 3G). The degree of simultaneous colocalization of $CD14^+$ and $CD34^+$ cells with vessels (Fig. 3F) was significantly less than either cell type considered by itself (Fig. 3G).

Co-injection of $CD34^+$ Cells With MSCs

In mice with I/R injury, the co-injection of $huCD34^+$ cells from diabetic donors along with murine MSCs resulted in a 2-fold increase in $CD34^+$ cell colocalization to retinal vasculature compared to $CD34^+$ cells alone, from 16 ± 2.4% to 32 ± 5.0% of vasculature, respectively (Fig. 4).

Effect of Peripheral Blood EPC Concentration on Vascular Association and Changes in Vascular Permeability With the Addition Peripheral Blood Diabetic EPCs or MSCs

There was no difference in cell association with vasculature by either $CD34^+$ or $CD14^+$ cells alone or in combination, and regardless of the disease state of the donor when cells were given at a dose of 1×10^3 per eye (Fig. 5A). By contrast, there was significantly higher vascular association when cells were given at a dose of 5×10^3 per eye ($P < 0.05$ compared to 1×10^3 cells per eye). Furthermore, at the higher cell dose the disease state of the donor also affected vascular association to a significant degree, with diabetic $CD34^+$ cells alone associating to a lesser degree than normal cells, while both diabetic $CD14^+$ cells and the combination of diabetic $CD34^+$ and $CD14^+$ cells showed more vascular colocalization than the respective normal cells ($P < 0.05$ compared to normal cells).

A difference in functional effect on vascular permeability was also seen with changes in cell dosing (Fig. 5B). When normal $CD34^+$ and $CD14^+$ cells were given together at a dose of 1×10^3 cells each, the measured leakage was statistically as great as when no cells were given (i.e., I/R injury alone) and was nearly three times greater than permeability measured in uninjured eyes ($P < 0.05$ compared to uninjured). By contrast, when normal cells were administered at a dose of 5×10^3 cells per eye, the measured permeability was statistically no different than uninjured eyes.

The administration of murine MSCs to I/R injured eyes had no effect on vascular permeability, with the amount of measured fluorescence leakage being statistically identical to that seen in injured eyes alone (Fig. 5B).

Ex Vivo Modification of Diabetic $CD34^+$ Cells

Diabetic human $CD34^+$ cells that were fucosylated using ACS101 reagent and protocol showed significantly greater vessel colocalization than untreated diabetic cells when given to mice subject to acute I/R injury (Figs. 6A, 6B). Fucosylation

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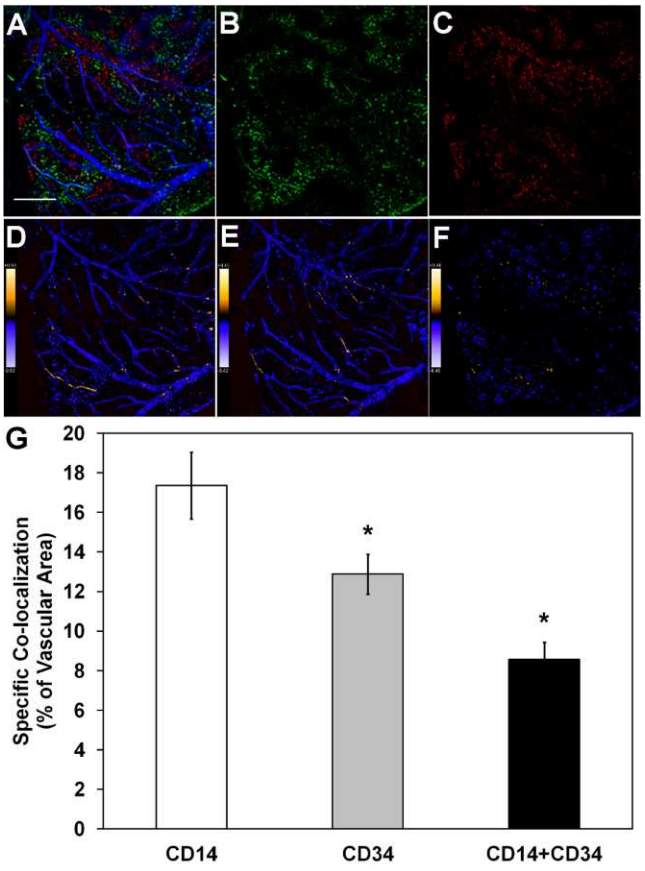

FIGURE 3. Diabetic CD34$^+$ and diabetic CD14$^+$ cells co-administered to STZ-diabetic recipient mice shows significantly lesser homing than either cell type alone. (**A–C**) Confocal image captures of a typical retina from an 11-month STZ-diabetic mouse show the qualitative differences in distribution and intensity of *green*-labeled CD34$^+$ cells (**B**) and *red*-labeled CD14$^+$ cells (**C**). Note that CD34$^+$ cells show a wider distribution than CD14$^+$ cells. (**D–F**) A visual representation of the quantitative morphometry for the three channels depicted in (**A–C**), and used to determine colocalization of cell types, where warmer colors indicate a greater probability of specific colocalization. (**D**) Colocalization of CD34$^+$ cells with vasculature; (**E**) colocalization of CD14$^+$ cells with vasculature; and (**F**) those areas where simultaneous localization of both CD34$^+$ and CD14$^+$ cells occurs with vasculature. Note the significantly smaller area of colocalized color in (**F**) than in either (**D**) or (**E**). *Scale bar* = 150 μm in (**A**) and applies to (**A–F**). Quantitative specific colocalization for each cell type is shown in (**G**). Note that while CD14$^+$ cells colocalized to vasculature to a greater degree than CD34$^+$ cells did when cells were given together, there was reduced homing to the vasculature. Furthermore, specific colocalization of both cells together was significantly less than either cell considered on its own. Each *bar* represents $n = 6$ animals; $^*P < 0.05$ compared to CD14$^+$ cells considered separately; $^{**}P < 0.05$ compared to CD34$^+$ cells considered separately. CD14$^+$ cells and CD34$^+$ cells were fluorescently labeled prior to administration using nanoparticles Qtracker 525 or Qtracker 625, respectively. Vasculature was labeled with rabbit anticollagen IV followed by goat antirabbit IgG conjugate to DyLight 649 and assigned to the *blue* channel by software during image capture.

resulted in a nearly 3-fold increase in specific co-localization ($P < 0.0001$, Fig. 6C). Fucosylation also improved the efficiency with which the CD34$^+$ cells of diabetic origin localized to vessels, as determined by the quantity of cells specifically colocalized versus the total of such cells present per field ($P < 0.001$, Fig. 6D).

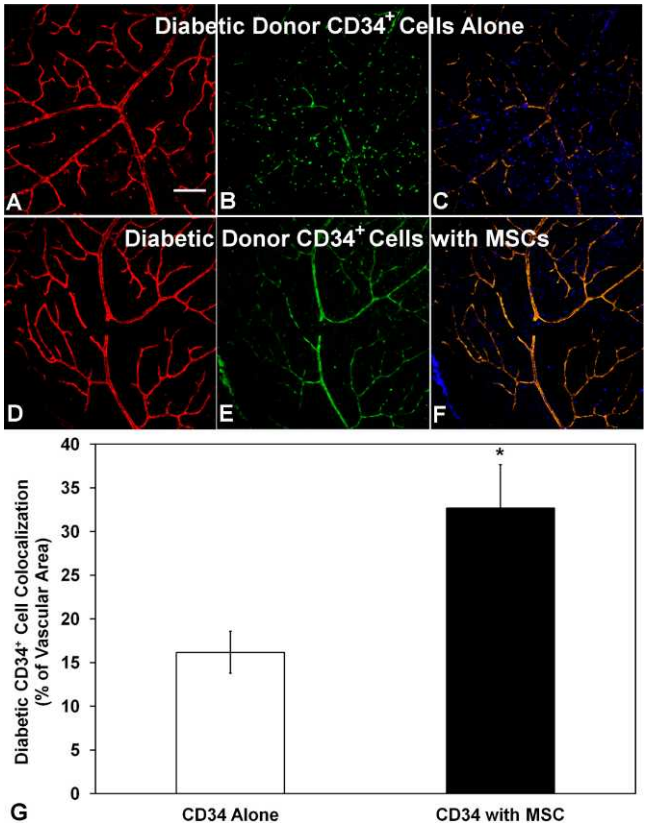

FIGURE 4. CD34$^+$ cells from diabetic donors colocalized to I/R-injured retinal vasculature to a greater degree when MSCs were co-administered. Representative confocal image captures (compressed z-series) are shown for vessel staining (**A**, **D**), CD34$^+$ cells (**B**, **E**), and colocalization (**C**, **F**) for diabetic CD34$^+$ cells given either alone or with equal numbers of MSCs, respectively. *Scale bar* = 50 μm in (**A**). The chart in (**G**) depicts the quantitative morphometry measurements of specific colocalization. Each *bar* represents an $n = 6$ animals; $^*P < 0.05$ compared to CD34$^+$ cells alone. Vasculature was labeled with rhodamine-conjugated *R. communis* agglutinin I, while cells of human origin (the injected CD34$^+$ cells) were labeled with mouse antihuman histone H1 followed by goat antimouse IgG conjugated to DyLight 488.

DISCUSSION

We previously demonstrated that bone marrow–derived hematopoietic progenitor cells injected intravitreally target the injured retinal vasculature.[12] We also showed that bone marrow–derived cells can differentiate into endothelial cells and exert trophic rescue activity in animal models of ischemic vascular damage[37,38] and diabetic retinopathy.[12] Bone marrow–derived progenitors home to areas of ischemia differentiate into microglia and endothelial cells and exert a paracrine-rescue effect stabilizing damaged retinal vasculature.[39]

The current study suggests that the homing of the CD14$^+$ cells is less adversely impacted by diabetes than is homing of CD34$^+$ cells. Diabetic CD34$^+$ cells demonstrate reduced endothelial nitric oxide synthase (eNOS) expression, decreased nitric oxide (NO) bioavailability, and a diminished migratory response to the chemokine stromal derived growth factor 1 (SDF-1α).[40] We previously showed that CD34$^+$ cells are exquisitely sensitive to oxidative injury[41] because increasing eNOS expression in diabetic cells by AVE3085 resulted in increased peroxynitrite levels and did not enhance NO-mediated functions in vitro and in vivo. This suggests that increasing the generation of NO was not sufficient to correct CD34$^+$ cell migration. Expression of Nox2, NADPH oxidase

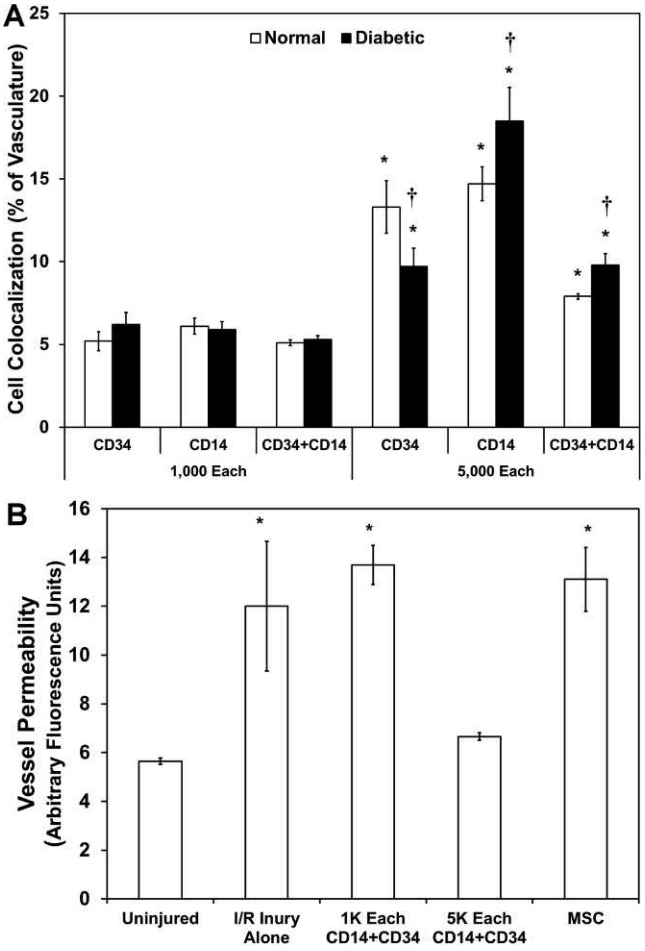

Figure 5. A minimum number of CD34$^+$ and/or CD14$^+$ cells were necessary to observe changes in vascular localization and effects on permeability, while MSCs had no functional effect. (**A**) When 1×10^3 CD34$^+$ and/or CD14$^+$ cells from either normal or diabetic donors were administered to I/R injured mouse eyes ($n = 5$ for each condition), no differences were seen in vascular colocalization with either cell type alone or in combination, regardless of the disease state of the donor. However, 5×10^3 cells, either alone or in combination, showed a significant increase in vascular association (*$P < 0.05$ compared to 1×10^3 cells). Additionally, with the higher cell dose, the disease state of the donor plays a role in colocalization with vasculature, since CD14$^+$ cells alone and the combined vascular association of CD34$^+$ and CD14$^+$ cells was greater when the donor was diabetic. However, diabetic CD34$^+$ cells alone at this dose—as with the higher dose (10×10^3) reported in Figure 4—were less able to colocalize with vasculature (†$P < 0.05$ compared to normal). (**B**) The dose of normal CD34$^+$ and CD14$^+$ cells was critical for a functional effect on vascular permeability. When I/R injured eyes ($n = 3$ per condition) were given 1×10^3 CD34 plus 1×10^3 CD14 cells together, the amount of leakage measured was the same as for I/R injured eyes that did not receive cells. When 5×10^3 of each cell type was administered together to I/R injured eyes, the measured leakage was no different than for uninjured eyes. On the other hand, administration of 10×10^3 mouse MSCs had no effect on the amount of vascular permeability resulting from I/R injury. *$P < 0.05$ compared to uninjured eyes.

activity, and superoxide levels were higher in diabetic than in nondiabetic CD34$^+$ cells and ex vivo NADPH oxidase inhibition in diabetic cells restored migratory function in vitro and enhanced their homing to ischemic retinal vasculature in vivo.[41] This work supports the key role of reducing oxidative stress in restoring CD34$^+$ cell function.

In this study, we demonstrate that CD14$^+$ myeloid progenitor cells, when injected into diabetic mice, home to areas of injury. We observed a different response of the CD14$^+$ cells and the CD34$^+$ cells to the retinal environment. The CD14$^+$ fraction, like CD34$^+$ cells, can respond to microenvironmental cues, regulate inflammatory cascades, respond to hypoxia, and influence the behavior of CD34$^+$ cells.[42] Marchetti et al.[24] demonstrated that during the hyperoxic phase of the oxygen-induced retinopathy model, CD14$^+$ cells induce angiogenesis, reducing the area of vaso-obliteration and associated tissue hypoxia. In that study, vascular regression, associated with endothelial cell death, was avoided by CD14$^+$ cell-induced up-regulation of anti-apoptotic and anti-oxidative genes and associated maintenance of retinal vasculature. CD14$^+$ cells differentiated into M2 macrophages, acquiring an anti-inflammatory phenotype.

Human monocytes differentiate into endothelial cells in vitro[4,15,17,43–45] and in vivo.[4,43,46,47] CD14$^+$ monocytes may be a major source of endothelial progenitors in the circulation.[4,44] Intravenous injection of CD14$^+$, CD34$^-$, or total peripheral blood mononuclear cells dramatically improved flow in the hindlimb ischemia model compared to untreated controls,[43] suggesting that injection of freshly isolated circulating CD14$^+$ cells may improve healing and vascular growth. Monocytes of diabetic origin appear to be less negatively affected by this disease than primitive progenitors, which are the source of CD34$^+$ cells.[42] Importantly, CD14$^+$ cells could provide a therapeutic option for people with diabetes because the function of CD34$^+$ cells in diabetes is often compromised.

To better understand the physiological events that promote the homing response of these cells, we examined their function in two distinct conditions, normal aging and diabetes. A comparison between aging and diabetes indicated the following: vascular changes associated with aging are sufficient to result in increased homing of precursor cells to the vessels (compared to young vessels), as evidenced by more healthy CD34$^+$ cells colocalizing with aged vasculature. However, the degree of such colocalization is less than that observed when diabetes is present. When the recipient animal has diabetes, there is greater colocalization of nondiabetic cells at 4 months of diabetes compared with 11 months suggestive that there are more homing signals present at 4 months rather than 11 months.

For these studies, CD34$^+$ cells of diabetic origin were isolated from donors with more than 10 years' duration of disease, with mild nonproliferative diabetic retinopathy. The diabetic subjects were specifically selected to be on minimal medication so as to lessen the impact of agents, such as thiazolidinediones[48] and statins,[49] which are known to improve CD34$^+$ cell function. The diabetic individuals were all on angiotensin converting enzyme (ACE) inhibitors but not on thiazolidinediones or statins. The reduction, but not absence, of colocalization of the diabetic human CD34$^+$ cells in the vessels of the aged diabetic mouse retina (Fig. 1J) may be explained by loss of homing signals.

In contrast to the CD34$^+$ cells of diabetic origin, the CD14$^+$ cells of diabetic origin can localize to putatively injured vessels to a greater degree than the CD14$^+$ cells of nondiabetic origin. We hypothesized that the CD14$^+$ cells of diabetic origin were activated and able to correct the dysfunction observed in the CD34$^+$ cells of diabetic origin. However, when co-injected, diabetic CD14$^+$ cells did not improve the migratory dysfunction of the CD34$^+$ cells, and the combination of cells showed less colocalization than either cell type alone.

Our work has limitations since we only examined in vivo homing (48 hours) and did not immunosuppress the diabetic animals to allow for a longer time course to examine incorporation of cells (as endothelial cells) into the vessels.

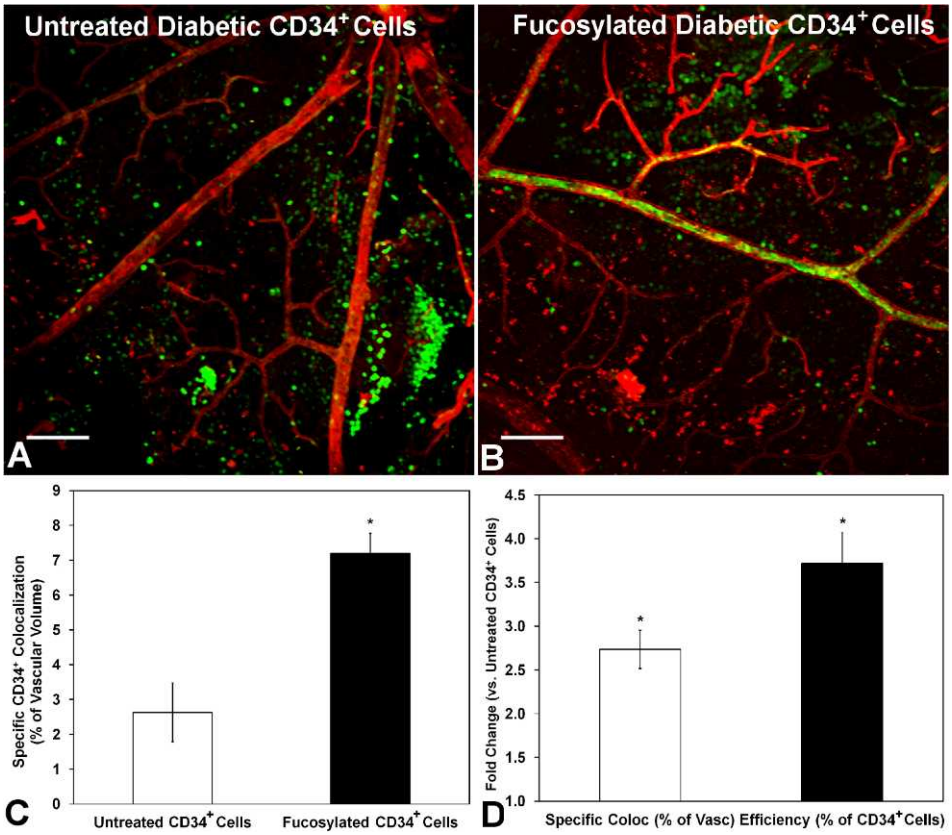

Figure 6. Ex vivo ASC-101–mediated enforced fucosylation of diabetic CD34$^+$ cells enhances colocalization of these cells to retinal I/R vasculature compared to unmodified cells following intravitreous injection. (**A**, **B**) Confocal image captures (compressed z-series) of retinas from I/R-injured mice that received either naïve diabetic CD34$^+$ cells or ex vivo fucosylated cells, respectively. Note the random and clumped distribution of the *green*-labeled cells under unmodified conditions (**A**) and the strong vascular association after fucosylation (**B**). *Scale bar* = 50 μm. Quantitative morphometry (**C**) of colocalization shows that fucosylation results in a nearly 3-fold increase in specific vascular colocalization of CD34$^+$ cells of diabetic origin (**D**), while the efficiency with which the cells colocalize—as a function of the area colocalized versus total area of labeled cells, is nearly 4-fold greater for fucosylated cells. Each *bar* represents $n = 8$ animals; $^*P < 0.0001$ for (**C**), $^*P < 0.001$ for (**D**). Vasculature was labeled with rhodamine-conjugated *R. communis* agglutinin I, while cells of human origin (the injected CD34$^+$ cells) were labeled with mouse antihuman histone H1 followed by goat antimouse IgG conjugated to DyLight 488.

However, we showed functional improvement as reduced permeability with the use of nondiabetic CD34$^+$ and CD14$^+$ cells. The significance of this work lies in its implications for autologous cell therapy in diabetics. Our results suggest that subsets as well as unfractionated whole bone marrow or peripheral blood cells would prove beneficial. Furthermore, future studies are needed to determine whether CD14$^+$ cells, specifically M2 cells, may have therapeutic benefit in diabetic subjects in need of revascularization procedures.

CD34$^+$ cells have been the cell type most frequently used in clinical trials. Unfortunately, their dysfunction in diabetics limits their utility for autologous cell therapy in that disease. Because diabetic CD34$^+$ cell dysfunction was not improved by the presence of CD14$^+$ cells, we tested whether the presence of MSCs would serve to correct diabetic CD34$^+$ cell dysfunction. MSCs have already been shown to have therapeutic benefit in correcting chronic inflammation.[50,51] While acute inflammation is critical to the survival of an organism, chronic inflammation typically leads to vascular and tissue pathology. MSCs are potent immune modulators, thus we tested these cells for their ability to correct CD34$^+$ cell dysfunction. The coinjection of MSCs with CD34$^+$ cells enhanced the migration and colocalization of CD34$^+$ cells with the retinal vasculature.

We also utilized a novel strategy to improve CD34$^+$ cell adherence. ASC-101 adds a missing fucose to the terminal ligand of cell surface P-selectin glycoprotein ligand-1, which allows the CD34$^+$ cells to bind to selectins found on retinal vascular endothelial cells, thus facilitating the first step in the homing process of CD34$^+$ cells to target tissue. This did not occur in nonfucosylated CD34$^+$ cells from the same diabetic donors.

Thus, patients with diabetic macular ischemia or patients with profound areas of retinal nonperfusion would have defective progenitor cell homing to damaged retinal vessels. This dysfunction can be corrected by ex vivo pretreatment of the CD34$^+$ cells with ASC-101 to enhance homing to damaged retinal vessels and begin the repair process, thus potentially preventing the downstream sequelae of neovascularization, retinal hemorrhage and vision loss. ASC-101 is currently in phase 1/2 clinical trial for enhancing the efficiency and homing of intravenously administered cord blood hematopoietic stem cells for bone marrow transplantation in patients with hematological malignancies following successful preclinical testing.[52] Interestingly, the process of bone marrow engraftment requires homing of hematopoietic stem cells from the blood to the bone marrow, a process that is similar to the "homing" that we show in the retina in the current studies.

In summary, the use of autologous progenitor cells represents a promising strategy for treatment of ocular diseases. In healthy individuals, these cells have the capacity to promote physiological repair and to reduce inflammatory processes associated with hypoxic damage in the retina. In

diabetes, one key population, $CD34^+$ cells, exhibit profound dysfunction that cannot be corrected by $CD14^+$ cells from the same patient, but can be enhanced by the use of MSCs or ex vivo manipulation of the cells with ASC-101. The potential utility of these cellular strategies for treatment of diabetic retinopathy and diabetic macular ischemia warrants further study.

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