Monocytes Form a Vascular Barrier and Participate in Vessel Repair after Brain Injury.

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Nonstandard abbreviations used: blood brain barrier (BBB); mouse brain endothelial cell (MBEC); human umbilical vein endothelial cells (HUVEC); human aorta endothelial cells (HAEC); endothelial-like monocytic cells (ELMC); transcellular electrical resistance (TCER); vonWillebrand Factor (vWF); Zonula Occludens 1 (ZO-1); Ulex europaeus agglutinin-1 (UEA-1), γ-glutamyl transpeptidase (γ-GT); alkaline phosphatase (ALP).
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Abstract

Subpopulations of bone marrow-derived cells can be induced to assume a number of endothelial properties in vitro. However, their ability to form a functional vascular barrier has not been demonstrated. We report that human CD14+ peripheral blood monocytes cultured under angiogenic conditions develop a number of phenotypic and functional properties similar to brain microvascular endothelial cells. These cells express the tight junction proteins zonula occludens 1 (ZO-1) and occludin and form a barrier with a trans-cellular electrical resistance (TCER) greater than 100 ohm cm² and low permeability to 4 kD and 20 kD dextrans. The TCER of the cellular barrier is decreased by bradykinin and histamine. We also demonstrate that these cells associate with repairing vasculature in areas of brain and skin injury. Our data suggest that CD14+ peripheral blood monocytes participate in the repair of the vascular barrier after brain injury.

Key Words: endothelial cell; progenitor cell; angiogenesis; blood brain barrier; monocyte
**Introduction**

Bone marrow-derived cells are important in the formation of new blood vessels in both physiologic and pathologic processes. Several populations of hematopoietic cells assume an endothelial phenotype when cultured under pro-angiogenic conditions. These include CD34+, Sca1+, CD133+, and CD14+ cells (1-19). Additionally, these cells incorporate into growing vasculature in experimental models (3, 20-29). This expanding body of evidence suggests a critical link between the ability of hematopoietic cells to adopt endothelial characteristics and the processes of blood vessel growth and repair.

Although there are certain common features of all endothelial cells, there is great specificity of function and heterogeneity of phenotype between different populations (30-32). A specialized form of endothelium makes up the microvasculature of the central nervous system and participates in the formation of the blood-brain barrier (BBB). The BBB is a complex micro-anatomical structure designed to closely regulate the influx and efflux of substances from the circulation to the brain parenchyma and vice versa. Several cell types, including endothelial cells, astrocytes, pericytes, and possibly neurons participate in the formation and maintenance of the BBB (33-40). Some of the features of brain microvascular endothelial cells which differentiate them from endothelial cells in other locations include the presence of numerous tight junctions, a decrease in the amount of pinocytosis, and the presence of enzyme systems involved in the transport of molecules in and out of the CNS (33, 39, 41-43).

*In vitro* systems for the culture of brain microvascular endothelial cells have been developed in which many of the properties of BBB endothelial cells can be maintained. Cultured brain microvascular endothelial cells can form a tight cellular barrier that develops a high trans-endothelial electrical resistance, impedes the passage of larger
molecules across the cellular layer, and has enzymatic activities associated with in vivo BBB endothelial cells (33, 38, 44-49). These in vitro systems also maintain some of the regulatory properties of the in vivo BBB such as an increase in barrier permeability mediated by vasoactive substances such as bradykinin and histamine (50-52).

We hypothesized that if cells of hematopoietic origin could express endothelial cellular markers, they may also be able to carry out other important endothelial functions. To that end, we now demonstrate that a population of peripheral blood-derived monocytes adopt endothelial characteristics including the formation of a regulated vascular barrier and migrate to damaged vasculature at sites of brain injury. These data suggest an important role for monocytes in repair of the cerebral vasculature.
Materials and Methods

Cell Culture. Leukopacks from consenting normal donors were obtained from the NIH blood bank or from the New Brunswick affiliated Hospitals blood bank. Mononuclear cells were isolated using histopaque-1077 density separation (Sigma, St. Louis, MO). CD14+ cells were isolated using anti-CD14 magnetic beads according to the manufacturer’s instructions (Miltenyi, Auburn, CA). CD14+ cells were then plated at a density of 1-2 x 10^7 cells / 80 cm^2 and cultured in endothelial basal media (EBM-2) with EGM-2-MV supplements (hydrocortisone, 5% FBS, VEGF, hFGF-b, IGF-1, hEGF, and ascorbic acid) (Cambrex Bio Science, Walkersville, MD) for 10-14 days at 37°C with 5% CO2 in a humidified atmosphere. Cells were then replated on 24mm diameter or 12mm diameter Corning Costar Transwell – Clear inserts, pore size 0.4 microns catalogue numbers 3450 (24mm) and 3460 (12mm) (Corning Incorporated, Corning, NY), that had been coated with a 5ng/ml solution of fibronectin (Sigma, Saint Louis, Mo.), on chambered microscope slides, or in tissue culture flasks. The ELMCs were cultured in M199 (Mediatech Inc., Herndon, VA) with 10% fetal calf serum (HyClone, Logan, UT) plus 50 ng/ml VEGF, 1 ng/ml basic FGF, and 1ng/ml IGF-1 (R&D Systems, Minneapolis, MN). Cells were grown an additional 10-14 days in this medium prior to cell staining, FACS analysis, or enzymatic analysis. Throughout this process the media was changed every 3 to 5 days. Whole human bone marrow was obtained from All Cells (Berkeley, CA) and plated at a density of 2 x 10^7 cells per 80cm^2. The human bone marrow cells were cultured in conditions identical to those used to generate ELMCs as described above. Mouse brain microvascular endothelial cells were isolated from Cr:NIH(S) (Swiss) mice (NCI, Frederick, MD) as previously described (38,44). Human umbilical vein endothelial cells (HUVEC) and human aorta endothelial cells (HAECs) were purchased from Cambrex (Walkersville, MD) and were used at passages 5 to 11.

Transcellular Resistance Experiments. After culture of cells on Transwell inserts, the TCER was measured using the EVOM voltohmmeter (World Precision Instruments, Sarasota, FL). For
experiments showing the effect of vasoactive substances, endothelial-like monocytic cells (ELMCs) with an established barrier were used. Bradykinin (Sigma, St. Louis, Mo.) to a concentration of 100 μM or histamine (Sigma, St. Louis, Mo.) to a concentration of 100 μM was added to both the upper and lower chambers and the TCER measured over time. DMSO was the vehicle for these drugs and was used as a negative control. The final DMSO concentration was 0.5%.

Diffusion of labeled dextran. Cells were cultured to confluence on Transwell Inserts. The media in both the upper and lower chambers of the Transwell was replaced with pre-warmed Hanks balanced salt solution. FITC-labeled dextran of either 4 kD or 20 kD (Sigma, St. Louis, Mo.) was added to the upper chamber of the Transwell to a concentration of 0.5 mg/ml and samples were taken of the buffer in the lower chamber over time. Fluorescence measurements were made using a Victor 2 1420 multilabel counter (Perkin Elmer, Boston, MA) and the concentration of dextran determined. Known concentrations of FITC-labeled dextrans were used to construct concentration curves for fluorescence for both 4kD and 20kD FITC-dextrans. The concentration of FITC-dextran in the upper and lower chambers was determined by fluorescence measurements of the chamber solutions using these curves. Permeability coefficients were calculated as previously described (38). Briefly, the permeability surface product (PS) was determined using the equation \( \frac{\Delta Cl}{\Delta t} \) where \( \Delta Cl \) is the incremental clearance volume and \( \Delta t \) is time. \( \Delta Cl \) is determined by multiplying the fraction of compound transferred from donor to acceptor compartment at a time point by the volume of the donor chamber. The permeability coefficient (P) was calculated using the equation \( \frac{PS}{S} = P \) where \( S \) is the filter surface area. To correct for the contribution of the filter, P was also determined for a fibronectin coated filter and the permeability coefficient for the cell layer alone was calculated using the equation \( \frac{1}{Pc} = \frac{1}{Pt} - \)
1/Pf where Pc is the permeability of the cell layer, Pt is the permeability of the total system and Pf is the permeability of the filter.

**Enzymatic Assays.** Cells in culture were rinsed three times with phosphate buffered saline and then scraped from the culture surface in a minimal volume of ice-cold lysis buffer (0.1% Triton X-100 in PBS). The cells were then stored at –80°C until enzyme activity was assayed. Just prior to assay, cells were rapidly thawed at 37°C and cell debris spun down at 10,000 x g for 10 minutes at 4°C. The supernatant was used in the enzyme assays. γ-Glutamyl transpeptidase (γ-GT) and alkaline phosphatase (ALP) activities were determined using diagnostic assay kits (Sigma, St Louis, MO). Protein concentration was determined using the Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA). Enzyme activities were expressed in U/mg protein. Human peripheral blood monocytes used in these experiments were >95% CD14 positive.

**Immunohistochemistry and FACS analysis.** The following antibodies were used: rabbit anti-von Willebrand factor (DAKO, Carpinteria, CA), rabbit anti-ZO-1 and anti-occludin (Zymed, South San Francisco, CA), mouse anti-CD14, CD11b, CD31 and rat anti-CD31 (BD Pharmingen, San Diego, CA), and labeled goat anti-rabbit, goat anti-rat, and goat anti-mouse secondary antibodies (Molecular Probes, Eugene, OR). Biotin labeled *Ulex europaeus* lectin1 (UEA-1) was purchased from Sigma (St. Louis, MO) and Texas red avidin from Vector Laboratories (Burlingame, CA). Cultured cells on glass slides or cytospin preparations of freshly isolated monocytes were fixed for 20 minutes at –20°C in 5% acetic acid in ethanol, blocked with 10% goat serum and 0.1% Triton X-100 in PBS then incubated at 4°C for 4-8 hours in 1:100 (ZO-1, occludin) or 1:200 (von Willebrand factor) of the primary antibody. Cells were washed and incubated in 1:1000 of the secondary antibody for 1 hour at 4°C, washed and mounted with vectashield with DAPI (Vector Laboratories, Burlingame, CA). For FACS analysis of CD14, CD11b, CD31, and occludin cells
were removed from culture using nonenzymatic cell dissociation buffer (Invitrogen, Carlsbad, CA), incubated with primary antibody at a concentration of 1μg per million cells for 30 minutes on ice, washed and incubated with secondary antibody at a concentration of 1μg per 10^6 cells. For FACS analysis of von Willebrand factor expression cells were removed from culture plates and fixed with 1% paraformaldehyde at 4°C for 20 minutes prior to staining. For tissue sections, mice were deeply anesthetized using a combination of ketamine (100mg/kg) and xylazine (4mg/kg) delivered intraperitoneally. The chest was opened and a small nick was made in the right atrium. The mice were then perfused through the left ventricle with 40ml of PBS. The brains were removed immediately and frozen in isopentane on dry ice. Brains were mounted in Tissue-Tec (Sakura Finetek USA, Torrence, CA) and 30 μM sections were cut and placed on glass slides. The tissue sections were then fixed on the slides in 100% methanol at –20°C for 15 minutes. The procedure for staining was as described for cultured cells. FACS analysis was performed and compared to corresponding isotype controls.

**DiI-Ac-LDL labeling.** Uptake of acetylated low-density lipoprotein (Ac-LDL) was performed on living cells with DiI labeled ac-LDL (Molecular Probes, Eugene, OR). DiI ac-LDL was added to a concentration of 10 μg/ml to the media and cells were returned to the tissue culture incubator for four hours. The cells were then washed three times with phosphate buffered saline and ac-LDL uptake was visualized using fluorescence microscopy.

**Bone Marrow Transplant Model.** FVB/N-TgN(TIE2LacZ)182Sato mice express β-galactosidase driven by the endothelial-specific TIE2 promoter. These mice were purchased from The Jackson Laboratory, Bar Harbor, ME. Mice were sacrificed and bone marrow collected from the femurs for use in the transplantation studies. Sublethally irradiated (350cGy) SCID/NCr mice (National Cancer Institute, Frederick, MD) were injected with 5 x 10^6 bone marrow cells via tail vein. One
month after transplantation mice were used in brain injury studies. Mice were anesthetized and under stereotaxic guidance a 30 gauge needle was inserted 2.5mm into the brain parenchyma 2mm lateral and 1mm anterior to the bregma. Mice were sacrificed 3-5 days later and sections through the injury site were analyzed. Injuries in five transplanted mice were analyzed. For evaluation of FITC-Albumin permeability, Cr:NIH (Swiss) mice (National Cancer Institute, Frederick, MD) were used. 0 to 12 days after stab injury, mice were deeply anesthetized and injected with 100 μl of a 10 mg/ml solution of FITC-labeled dextran (Sigma, St. Louis, MO) via tail vein. Ten minutes after tail vein injection, mice were perfused through the left ventricle with 40 ml of phosphate buffered saline. The brains were removed, frozen, fixed, and stained as described below.

Incorporation of ELMCs into repairing vasculature after brain injury. Cold injury was performed on SCID mice as described previously (53). Briefly, mice were anesthetized using a combination of ketamine (100mg/kg) and xylazine (4mg/kg) IP. The skull was then exposed. Under stereotaxic guidance a metal rod 4mm in diameter that had been cooled to –80°C in dry ice was applied to the skull 2mm lateral and 1mm anterior to the bregma. The probe was applied for 30 seconds. ELMCs, freshly isolated human CD14+ peripheral blood mononuclear cells, or CD14-depleted human peripheral blood mononuclear cells were labeled with Cell Tracker DiI (Molecular probes, Eugene, OR) according to the manufacturer’s instructions. Twenty-four hours after injury 10⁶ DiI labeled human cells were injected into each mouse via tail vein. Mice were sacrificed at either 5 days or 4 weeks after the injury and tissue sections were analyzed.

Incorporation of ELMCs into repairing vasculature after skin injury. A skin biopsy was performed on SCID mice. Briefly, mice were anesthetized using a combination of ketamine
(100mg/kg) and xylazine (4mg/kg) IP. The back was then shaved. A circular skin biopsy punch was used to make an injury on the back. ELMCs were labeled with Cell Tracker Dil (Molecular probes, Eugene, OR) according to the manufacturer’s instructions. Twenty-four hours after injury 10⁶ Dil labeled human cells were injected into each mouse via tail vein. Mice were sacrificed 5 days after the injury and tissue sections were analyzed.

Animal Experiments: All procedures followed in the animal experiments were in accordance with NIH or UMDNJ/RWJMS institutional guidelines and had been approved by the appropriate institutional animal care and use committee.

Statistical Analysis: Results are expressed as mean +/- SEM. Statistical analysis was assessed using the Student t-test. The lower limit of significance was taken as p<0.05.

Results

CD14+ peripheral blood monocytes form a cellular layer with high electrical resistance in vitro. Various models have been used to study the permeability characteristics of brain microvascular endothelial cells (38, 45-50, 54-56). One well-defined system, the Transwell (Corning), consists of a filter dividing upper and lower chambers. Cells can be cultured on the filter and the movement of drugs and other molecules from upper (apical) to lower (basolateral) chamber measured. The electrical resistance of a cell layer or trans-cellular electrical resistance (TCER) can also be measured (38, 47, 50). We utilized
this model system to identify a population of human peripheral blood-derived cells with the capacity to generate a cellular barrier with a high trans-cellular electrical resistance similar to brain microvascular endothelial cells. Cells from human bone marrow and peripheral blood were cultured in endothelial growth media with microvascular supplements (EGM-2, Clonetics) for 10 days and then transferred to the Transwell system. The cells were then grown in the presence of various angiogenic cytokines and other growth factors. Cultures were screened for the formation of a tight cellular barrier using TCER measurements. Human peripheral blood mononuclear cells consistently formed a cellular barrier with a high TCER when grown in the presence of VEGF, bFGF, and IGF1. By contrast, human bone marrow cells cultured under the same conditions adopted a mesenchymal morphology and did not generate a high TCER.

Preliminary immunohistochemical data suggested that the cells responsible for this TCER phenotype were of the monocyte/macrophage lineage (data not shown). To confirm these observations we performed subsequent experiments using CD14+ monocytes. In multiple experiments, monocytes were consistently able to produce a TCER of over 100 ohm cm\(^2\) compared to CD14-depleted human peripheral blood mononuclear cells which did not form a confluent monolayer and did not generate resistances over 20 ohm cm\(^2\). (Figure 1A). The TCER seen using peripheral blood mononuclear cells was similar to that seen with mouse brain endothelial cells when used in the same system. Additionally, human umbilical vein endothelial cells (HUVECs) as well as human aorta-derived endothelial cells did not form a TCER under similar culture conditions (Figure 1A). Based on these observations we have referred to the CD14+
human peripheral blood cells grown under these conditions as endothelial-like monocytic cells (ELMCs).

The ELMC barrier has low permeability to high molecular weight dextrans. The barrier formed by the ELMCs also has a low permeability to larger molecules. FITC-labeled dextrans (4kD or 20kD) were applied to the upper chamber of the Transwells on which ELMCs, HUVECs, or mouse brain endothelial cells were grown and samples of the lower chamber were taken at 30 and 60 minutes. The permeability of ELMCs for 4kD dextran (Pc = 3.8 +/- 1.1 x 10^-6 cm/s) was significantly lower than the permeability of HUVECs for 4kD dextran (5.5 +/- 1.3 x 10^-6 cm/s) p<0.05. This was also true for 20kD dextran (ELMCs Pc=2.23 +/- .1 x 10^-6 cm/s vs HUVEC Pc=3.8 +/- .4 x 10^-6 cm/s; p<0.05). The permeability of ELMCs to both 4kD and 20kD dextran was similar to mouse brain endothelial cells (Figure 1B,C).

The permeability of cellular barriers, including the BBB, can be modulated by a number of vasoactive compounds (52). In order to determine if the barrier formed by ELMCs could be modulated in a similar way, we evaluated the effect of histamine and bradykinin on the TCER of ELMCs. Confluent ELMC cultures on transwells that had developed a high resistance barrier were prepared. Histamine, bradykinin, or vehicle was applied to both the upper and lower chambers and the TCER was measured over time. Bradykinin caused a decrease in the TCER by 20% over 30 minutes and histamine a decrease of 30% over 30 minutes (Figure 2). The magnitude of the decrease in TCER is comparable to the reported effect of bradykinin and histamine in other in vitro BBB systems (51, 52).
The activity of several enzyme systems is elevated in BBB-derived endothelial cells compared to endothelial cells from other locations (32, 33, 38, 47, 55). Thus, we measured the activity of γ-glutamyl transpeptidase and alkaline phosphatase in ELMCs in order to determine if ELMCs had elevated expression of these BBB-associated enzymes. γ-glutamyl transpeptidase activity was approximately 3-fold higher in ELMCs compared to monocytes or HUVECs and was similar to the level of γ-glutamyl transpeptidase activity seen in mouse brain endothelial cells (Figure 3A). By contrast, ELMCs did not have increased alkaline phosphatase activity compared to freshly isolated monocytes or HUVECs and had significantly lower alkaline phosphatase activity than mouse brain endothelial cells (Figure 3B).

*Human peripheral blood monocytes express endothelial markers when cultured with pro-angiogenic cytokines.* In order to better define the cellular phenotype of the ELMCs once they had formed an intact barrier, we examined their immunophenotype. ELMCs express the endothelial specific marker von Willebrand factor and were stained with the endothelial specific lectin *Ulex europaeus* agglutinin-1 (UEA-1) (Figure 4). Additionally, the cells continued to express markers common to cells of both the monocytic and endothelial lineages such as acetylated LDL uptake and CD31 staining (Figure 4). They also retained the expression of the monocyte markers CD14 and CD11b (Figure 4). ELMCs also express the tight junction proteins ZO-1 and occludin, whereas freshly isolated monocytes did not (Figure 4). Taken together, these data demonstrate that ELMCs have an immunophenotype intermediary between BBB-derived endothelial cells and monocytes.
Bone marrow-derived cells localize to the repairing vasculature after brain injury. It has been demonstrated that bone marrow-derived cells can incorporate into areas of new vasculature formation in both pathologic and physiologic conditions (2,3, 20-29). A bone marrow transplant was performed on SCID mice using donor marrow from FVB/N-TgN (Tie2-LacZ)182Sato mice. These mice express β-galactosidase driven by the Tie2 promoter specifically in endothelial cells. A stab injury was performed on the mice one month following transplantation. Three to five days after the injury the mice were sacrificed and sections of the injured brain were stained for β-galactosidase and endothelial markers. Bone marrow-derived cells were present, and incorporated into the vasculature in the area of injury (Figure 5). Thus, hematopoietic cells appear to participate in repair of the vasculature in the brain.

Labeled ELMCs localize to vasculature at sites of injury. To more directly demonstrate the homing of ELMCs to the repairing cerebral vasculature two distinct injury models were used. Initial experiments used a stab injury model in SCID mice. Twenty-four hours after injury DiI labeled human ELMCs were injected via tail vein and three to five days later the animals were sacrificed and brains sectioned and stained for endothelial markers. DiI labeled cells localized to repairing vasculature at sites of brain injury and were seen in a perivascular location (Figure 6A-C).

To more completely assess the time course and microanatomical location of the ELMCs after injury, a cold injury model was employed. One day after cold injury, labeled human ELMCs, freshly isolated monocytes, or freshly isolated CD-14 depleted
cells were injected via tail vein. Mice were sacrificed at either 5 days or four weeks after injury. Five days after injury labeled ELMCs were present at the injury site. The majority of cells were located at the periphery of the injury with only rare labeled cells seen in areas of uninjured brain (Figure 6D). Labeled cells could be found in both a perivascular location and in positions more distant from vessels. A similar pattern was seen with uncultured human CD14+ cells (Figure 6D). However, only rare labeled CD-14 depleted cells were apparent at the injury site. At four weeks after injury neither labeled ELMCs nor newly isolated CD14+ cells were present at the injury site, suggesting that these cells are present transiently during vessel repair.

In order to determine the distribution of injected ELMCs to other organs the liver, spleen, and lungs of experimental animals were collected. Labeled ELMCs were present in the spleen but were not observed in the lungs or liver (data not shown).

The ability of ELMCs to form a high resistance barrier suggests that they may have a specific role in brain vasculature. We therefore investigated the ability of ELMCs to home to a site of injury outside of the brain. Skin biopsies were performed on SCID mice and one day after biopsy labeled ELMCs were injected via tail vein. The mice were sacrificed five days after injury and the skin injury site was sectioned. Labeled ELMCs could also be seen in the area of the skin injury (Figure 6E). This suggests that the ELMC phenotype is not specific to the central nervous system.

Discussion

Recent studies have documented the critical role of hematopoietic cells in vascular growth and repair. Bone marrow-derived endothelial-like cells incorporate into
vasculature following ischemic injury to various organs including the heart (57-60), limb (3, 7, 20, 61, 62), retina (21), and brain (29) and also participate in the formation of tumor vasculature (2, 3, 20, 22, 25). The magnitude of this contribution has been estimated to be between 2 and 20% (26, 62, 63).

Two broad categories of circulating cells with the capacity to assume endothelial traits have been described (63,64). Cells expressing hematopoietic progenitor markers such as CD34 and CD133 with a high proliferative capacity and the ability to differentiate into mature endothelial cells have been variously categorized as endothelial progenitor cells (EPC)(24,15,13) and outgrowth endothelial cells (64). EPCs express endothelial-specific proteins such as VE-Cadherin and do not express myeloid markers such as CD45 and CD14. They also have the capacity to incorporate into growing vasculature (7). Cells of the monocyte/macrophage lineage also express endothelial markers when cultured in the presence of pro-angiogenic cytokines (6-8, 12, 16, 19,64). However, as our data as well as that of others show, they maintain expression of monocyte markers such as CD14 and CD45 (6,19,64,67). The continued expression of myeloid proteins implies that the cells do not trans-differentiate into mature endothelial cells, but rather take on specific endothelial characteristics while remaining inherently monocytic cells. These findings suggest an expanded functional repertoire for monocytes.

Several lines of evidence implicate endothelial-like monocytes in angiogenesis. In addition to expressing endothelial markers, monocytes cultured with proangiogenic cytokines localize to injured vasculature after intravenous injection (7) and produce angiogenic growth factors that may act as a stimulus for endothelial cell proliferation.
They may also play an important role in “drilling” tunnels for new vasculature (65, 66) and are able to adhere to injured vessel walls and accelerate re-endothelialization (68). Our data add participation in a physiologically regulated blood/tissue barrier to the possible role of monocytes and macrophages in vessel growth.

The ability of CD14+ cells to produce a barrier with a low permeability is unexpected. While comparable to the in vitro barrier formed by mouse brain microvascular endothelial cells, there are important differences between these two cell types. ELMCs form a stringent barrier in the presence of concentrations of VEGF that would be expected to disrupt the BBB (39, 51) and have a lower level of alkaline phosphatase expression. Other in vitro blood brain barrier studies use manipulations such as serum starvation and co-culture with astrocytes to obtain significantly higher TCER (34, 35, 38, 46, 47). The response of the ELMC barrier to similar conditions remains an area for further study. Additionally, ELMCs localize to sites of injury both inside and outside of the CNS, suggesting that the role of ELMCs in vascular repair is not limited to the brain.

The potential pitfalls of an immunocompromised murine model necessitate that the in vivo data be interpreted with caution. However, taken together, the localization of ELMCs to injury sites in a time frame corresponding to reformation of the BBB, their presence in a perivascular location, and in vitro evidence demonstrating the ability of these cells to form a cellular barrier suggests that they are involved in repair of the vasculature after injury. The homing of un-manipulated CD14+ cells to injury sites implies that peripheral blood monocytes localize to injury sites initially and are then exposed to proangiogenic stimuli and develop endothelial-like characteristics. The
absence of injected ELMCs at the injury site after one month suggests that their function is transient.

In summary, we show that peripheral blood monocytes form a cellular barrier *in vitro* with a high TCER and low permeability to larger molecules that is responsive to physiologic stimuli such as bradykinin and histamine. We also provide evidence that monocytes may function *in vivo* in this capacity by demonstrating that cells from both transplanted bone marrow and exogenously cultured ELMCs localize to repairing vasculature. Thus, a previously unrecognized function of monocytes (or a monocyte subpopulation) may be their participation in the formation of a blood-tissue barrier, a function that has traditionally been within the exclusive domain of endothelial cells. More work is required to better define the importance of this phenotype in normal physiology.

**Figure Legends**

**Figure 1**

*Human peripheral blood monocytes form a low permeability barrier in culture.*  (A) CD14+ human peripheral blood monocytes cultured on Transwells form a high resistance barrier similar to mouse brain microvascular endothelial cells (MBEC) while CD14-depleted human peripheral blood mononuclear cells do not. Human umbilical vein endothelial cells (HUVECs), and human aorta endothelial cells (HAEC) do not develop a high transcellular resistance under the same culture conditions. (n=3-4 for each cell type) p <0.05 (CD14+ □; MBEC ▲; HAEC ○; HUVEC ■; CD14-depleted ×). (B,C) The permeability of ELMCs to FITC-labeled 4kD (B) and 20kD (C) dextran is lower than permeability of HUVECs and similar to the permeability of
mouse brain endothelial cells (MBEC). Cells were cultured on transwell inserts and the movement of FITC-labeled 4kD and 20kD dextrans through the cell layer was monitored and used to calculate permeability coefficients (HUVEC \( \Delta \); ELMC \( \bullet \); MBEC \( \square \) ) \( n=3 \). The permeability surface product (PS) was determined using the equation \( \Delta CI/\Delta t \) where \( \Delta CI \) is the incremental clearance volume and \( \Delta t \) is time. The permeability coefficient (P) was calculated using the equation \( PS/S=P \) where \( S \) is the filter surface area. To correct for the contribution of the filter, P was also determined for a fibronectin coated filter and the permeability coefficient for the cell layer alone was calculated using the equation \( 1/Pc = 1/Pt – 1/Pf \) where \( Pc \) is the permeability of the cell layer, \( Pt \) is the permeability of the total system and \( Pf \) is the permeability of the filter. The permeability of ELMCs for 4kD dextran (\( Pc = 3.8 +/- 1.1 \times 10^{-6} \) cm/s) was significantly lower than the permeability of HUVECs for 4kD dextran (\( Pc = 5.5 +/- 1.3 \times 10^{-6} \) cm/s) \( p<0.05 \). This was also true for 20kD dextran (ELMCs \( Pc = 2.23 +/- .1 \times 10^{-6} \) cm/s vs HUVEC \( Pc = 3.8 +/- .4 \times 10^{-6} \) cm/s; \( p<0.05 \)). This experiment was performed twice with similar results.

Figure 2

The cellular barrier formed by ELMCs is responsive to bradykinin and histamine. A decrease in trans-cellular electrical resistance (TCER) is seen with the application of histamine (A), or bradykinin (B) to endothelial-like monocyctic cells cultured on transwells (bradykinin or histamine \( \bullet \); vehicle \( \square \) ). Experiments were done three times each with \( n = 3 \). TCER values for samples after the application of bradykinin or histamine are significantly lower than the controls at each time point (\( P<0.05 \)).

Figure 3

ELMCs have high \( \gamma \)-glutamyl transpeptidase (\( \gamma \)-GT) activity. \( \gamma \)-GT activity is significantly higher in ELMCs than HUVECs or freshly isolated monocytes (\( P<0.05 \)) (Indicated by an *). However,
ELMCs do not have increased alkaline phosphatase (ALP) activity compared to freshly isolated monocytes or HUVECs. \(\gamma\)-GT (A) and ALP (B) activity of freshly isolated monocytes (monocytes), endothelial-like monocytic cells (ELMC), human umbilical vein endothelial cells (HUVEC), and mouse brain endothelial cells (MBEC) were compared (n=3). Results are expressed as enzyme activity per mg of protein. These experiments were performed four times with similar results.

**Figure 4**

*Immunohistochemical and immunocytochemical analysis of ELMCs.* (A) Freshly isolated human peripheral blood CD14+ monocytes, endothelial-like monocytic cells (ELMC), human umbilical vein endothelial cells (HUVEC), and mouse brain endothelial cells (MBEC) were evaluated for uptake of Dil-labeled Acetylated LDL (Dil Ac-LDL), expression of von Willebrand Factor (vWF), Zonula Occludens-1 (ZO-1), and Occludin by immunohistochemistry. ELMCs, HUVECs, and freshly isolated monocytes were also evaluated for binding of the endothelial-specific lectin *Ulex europaeus* agglutinin-1 (UEA-1). (B) FACS analysis of freshly isolated monocytes, ELMCs, and HUVECs for expression of CD14, CD11b, CD31, vWF, and occludin was performed.

**Figure 5**

*Bone marrow-derived cells incorporate into the repairing vasculature in the injured brain.* Stab injury to the brain was performed on Swiss mice. 0 to 12 days after injury the mice were injected intravenously with FITC-labeled albumin and perfused with PBS 10 minutes after injection. Sections through the area of brain injury show leakage of FITC-labeled albumin from the vasculature that is no longer evident by day 12 (A). Bone marrow transplants were performed on SCID mice using marrow from FVB/N-TgN (Tie2-LacZ)182 Sato mice which express \(\beta\)-galactosidase specifically in endothelial cells. One month after transplant, stab injury of the brain
was performed. Three to five days after the injury the animals were sacrificed and sections through the injured area were stained with anti-β-galactosidase (green) and the endothelial marker CD31 (red). Fluorescence microscopy shows incorporation of β-galactosidase expressing cells into the repairing vasculature. Scale bars represent 20μm (B) and 100 μm (C).

**Figure 6**

*ELMCs participate in vascular repair after brain injury.* Stab brain injury was performed on SCID mice and DiI-labeled ELMCs were administered to mice via tail vein 24 hours after injury. 3-5 days later the mice were sacrificed and sections through the area of injury were stained for the endothelial markers CD105 and ZO-1. DiI-labeled ELMCs (red) and vascular endothelial cells stained with CD105 (green) in (A) and (B) and ZO-1 (green) (C) are shown. Labeled ELMCs can be seen in a perivascular location. Scale bars are 20μm (A,B) and 10μm (C).

ELMCs are located at the periphery of the injury site in a cold brain injury. Cold injury was performed on SCID mice. DiI-labeled human ELMCs, freshly isolated CD14+ monocytes, or CD14 depleted peripheral blood mononuclear cells were injected via tail vein 24 hours after injury. Mice were sacrificed five days after injury and sections were stained for GFAP (D). H&E sections of the corresponding injury site are shown for comparison (D). Red labeled uncultured monocytes and ELMCs can be seen at the periphery of the injury site. CD-14 depleted cells are not present. Reactive astrocytes around the injury site are stained with GFAP (green).

ELMCs will localize to injuries at non-CNS sites. Skin punch biopsy was performed on the backs of SCID mice. DiI-labeled ELMCs were administered to mice via tail vein 24 hours after injury. Five days after injury the mice were sacrificed and sections through the area of injury were obtained. DiI-labeled ELMCs (red) (E). Nuclei are labeled with DAPI.
References


circulating peripheral blood endothelial progenitor cells in the rhesus macaque model.

_Hum Gene Ther_ 13:2041-2050.


Figure 1A
Figure 1
Figure 3A

[Bar chart showing γGT activity (U/mg) for HUVEC, MBEC, ELMC, and monocyte cells.]

Figure 3B

[Bar chart showing ALP Activity (U/mg) for HUVEC, MBEC, ELMC, and monocyte cells.]
**Figure 4A**

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<th>ELMC</th>
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Figure 4B

Fluorescence intensity
Figure 5

A

Day 0  Day 4  Day 8  Day 12

B

C

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Figure 6
Figure 6D

Uncultured monocytes  ELMC  CD14 depleted
Figure 6E
Monocytes form a vascular barrier and participate in vessel repair after brain injury

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