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α 4 Integrin/FN-CS1 Mediated Leukocyte Adhesion to Brain Microvascular Endothelial Cells Under Flow Conditions

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Abstract

Insights into sequential leukocyte-endothelial interactions during leukocyte trafficking have been obtained through experiments using human umbilical vein endothelial cells (HUVEC) under flow conditions. To investigate leukocyte-brain endothelial cell interactions, we developed a dynamic *in vitro* system, using Transfected Human Brain Microvascular Endothelial Cells (THBMEC) and a parallel plate flow chamber. Human peripheral blood mononuclear cells (PBMC) were perfused across confluent THBMEC cultures at a velocity that approximates the rate found in human brain capillaries. Leukocyte-THBMEC interactions were visualized by phase-contrast microscopy, and images were captured on a CCD camera. To simulate inflammatory conditions, we activated THBMEC with the inflammatory cytokines tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ), which up-regulated chemokine and adhesion molecule expression in THBMEC without affecting the distribution of immunoreactivity for tight junction-associated proteins. PBMC adhesion was enhanced by cytokine-mediated activation of THBMEC. G protein-coupled receptor (GPCR) activation was essential for leukocyte-THBMEC interaction, as pertussis toxin (PTX) treatment of PBMC abrogated PBMC adhesion to activated THBMEC. The anti- α 4 integrin antibody, natalizumab, infused into MS patients, significantly reduced the adhesion of their *ex-vivo* PBMC to activated THBMEC under flow conditions. Further study showed that alternatively spliced fibronectin containing the CS1 region (FN-CS1), but not Vascular Cell Adhesion Molecule type 1 (VCAM-1), was the ligand of α 4 integrin on activated THBMEC. Blocking FN-CS1 abrogated PBMC adhesion on activated THBMEC, while anti-VCAM-1 antibodies had no effect. These results established a novel *in vitro* dynamic BBB model. We also demonstrated the dependence of leukocyte-endothelial interactions in this model on α 4 integrins and FN-CS1.

Keywords

chemokines; α 4 integrin; leukocyte; blood brain barrier; adhesion; shear stress; Multiple Sclerosis

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1. Introduction

Elucidating mechanisms of leukocyte migration across the blood brain barrier (BBB) may be useful for understanding the pathogenesis of disorders including multiple sclerosis (MS), brain and spinal cord trauma, stroke, and HIV infection (Chaudhuri et al., 2008; Danton and Dietrich, 2003; Lossinsky and Shivers, 2004; McCandless et al., 2008; Wang et al., 2008). Components that subserve leukocyte-endothelial cell interactions define the molecular pathways that regulate leukocyte trafficking.

Insights into sequential leukocyte-endothelial interactions have been obtained through experiments using human umbilical vein endothelial cells (HUVEC) under flow conditions (Schreiber et al., 2007; Burns et al., 2000). The step-wise interactions between leukocytes and endothelial cells are governed by adhesion molecules, chemokines, and chemokine receptors. Chemokine engagement of cognate G protein-coupled receptor (GPCRs) activates leukocyte integrins through inside-out signaling, resulting in the leukocyte adhesion to endothelial cells (Doring et al., 2007; Hartmann et al., 2008; Luster et al., 2005; Man et al., 2008; Schreiber et al., 2007; Shulman et al., 2006; Woolf et al., 2007). Unexpectedly, flow conditions promote, rather than antagonize, leukocyte-endothelial interactions (Ransohoff et al., 2007). As one example, T cells encountering apically-presented CXCL12 underwent robust LFA-1-dependent transmigration across HUVEC toward abluminal CCL5 under shear stress, in contrast to negligible transmigration under static conditions (Schreiber et al., 2007).

Leukocytes entering the CNS parenchyma must cross the BBB (Bechmann et al., 2007; Engelhardt and Ransohoff, 2005). The barrier function of the BBB relies on highly specialized brain microvascular endothelial cells, which lack pinocytotic vesicles and fenestrae but possess intercellular tight junctions. These features limit transcellular and paracellular movement of cells and molecules (Man et al., 2007). Mechanisms of leukocyte migration across the BBB are incompletely understood. Transwell cultures of brain endothelial cells have been used to study leukocyte trafficking into the brain (Callahan et al., 2004; Kraus et al., 2004; Luster et al., 2005; Mahad et al., 2006; Man et al., 2008; Ubogu et al., 2006). However, the lack of shear stress limits the interpretation of the results of such studies. The current report describes our progress in developing an *in vitro* BBB model using human brain microvascular cells and a parallel plate flow chamber to study leukocyte-brain endothelial cell interactions under flow conditions. The current paper focuses in particular on leukocyte adhesion to brain microvascular endothelial cells under flow. Analyses of the earlier stages of leukocyte/endothelial interaction, tethering and rolling, are ongoing.

Anti- $\alpha 4$ integrin antibodies (natalizumab; NTZ) is an FDA approved treatment for MS. Although it was designed to block $\alpha 4$ integrin-mediated leukocyte-endothelial interactions, this effect has not been directly evaluated in MS patients receiving NTZ. Using a static BBB model, we found that NTZ infusion to MS patients inhibited leukocyte migration across BBB, and that this blockade was reversed by removing NTZ with plasmapheresis (Khatri et al., 2008). We extended these results in the present study by examining NTZ effects under flow conditions.

2. Materials and Methods

2.1. Patients

Eligible patients were 18 to 50 years of age, and were diagnosed with relapsing remitting MS (RRMS). All the patients were free of relapse within the last month. MS patients (n=9) were recruited from the Mellen Center for Multiple Sclerosis Treatment and Research at the Cleveland Clinic. Before assay, patients had received at least three courses of NTZ, following the recommendations outlined in the US Food and Drug Administration-mandated Tysabri Outreach Unified Commitment to Health (TOUCH) program, and tested negative for anti-

natalizumab antibodies. Four additional MS patients had been treated with interferon β -1a 30 mcg IM weekly for at least 12 months. All patients gave written informed consent, and all study protocols were approved by the Cleveland Clinic Institutional Review Board. Patients and healthy controls had not received corticosteroid within the last three months. Clinical data were collected by an investigator blinded to the leukocyte adhesion assay, with the oversight of an independent monitor (Table 1).

2.2. Cell culture and PBMC isolation

THBMEC are adult human brain microvascular endothelial cells transfected and immortalized with a plasmid containing simian virus 40 large T antigen (SV40-LT) as previously described (Mahad et al., 2006; Man et al., 2008; Stins et al., 1997). THBMEC were grown in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 10% Nu-Serum, 2 mM glutamine, 1 mM pyruvate, essential amino acids, and vitamins. PBMCs were isolated from fresh whole heparinized blood from healthy volunteers or MS patients by density centrifugation, using Lymphocyte Separation Medium (Mediatech, Herndon, VA) as previously described (Mahad et al., 2006; Man et al., 2008; Stins et al., 1997). PBMCs were resuspended at 5×10^5 cells/ml in transendothelial migration (TEM) buffer (RPMI 1640 without phenol red + 1% bovine serum albumin). The research protocol was approved by the local institutional review board, and signed informed consent was obtained from all donors studied.

2.3. THBMEC activation (aTHBMEC) and PBMC adhesion assay

THBMEC were cultured on a 35mm collagen-coated Corning tissue culture dish to subconfluence, followed by activation with TNF- α 10 u/ml and IFN- γ 20 u/ml (R&D systems, Minneapolis, MN) or control media for 24 hours. THBMEC cultures were washed with TEM buffer twice, and immediately assembled with a parallel plate flow chamber and mounted on the stage of a Leica DMI3000 B inverted phase contrast microscope (Leica Microsystems Inc. Bannockburn, IL). This allowed for the study of leukocyte-endothelial interactions from a top-down view under a flow rate of 9ml/hr, calculated according to the size of the chamber and the velocity in human brain capillaries, which is 1mm/sec (Hudetz et al., 1996). This velocity was generated by a Medfusion 3010a syringe pump (Smiths Medical, St. Paul, Minnesota). Since the TEM buffer we used has a lower viscosity than the blood, the shear stress we used, which is 0.25dyn/cm², should be lower than the shear stress in brain capillaries. In pilot studies, higher flow rates decreased adherent cell numbers. The experiments were performed at 37°C. Cell interactions on a field of 0.15 mm² were visualized with a 20 $\times$ objective. Cell images were captured 2 frames per second with a QICAM fast 1394 CCD camera (QImaging, Pleasanton, CA) during 20 minutes of shear application. Cell images were manually quantified by analysis of the video.

For G_o/i₁ inhibition assay, PBMC were treated with pre-activated pertussis toxin (PTX) at 100 ng/ml for 10 min, followed by perfusion across aTHBMEC.

For experiments using antibodies to block endothelial adhesion molecules, aTHBMEC were incubated with mouse anti-human FN CS-1 IgM (clone P1F11, Santa Cruz Biotechnology, Santa Cruz, CA), mouse IgM Isotype (clone MOPC 104E, Sigma-Aldrich Inc. St. Louis, MO), neutralizing rat anti-mouse/human VCAM-1 IgG1 (clone M/K-2, Santa Cruz Biotechnology, Santa Cruz, CA) antibody, mouse anti-human VCAM-1 IgG1 (clone 4B2, R&D, Minneapolis, MN) antibody that have been previously proved as functionally neutralizing antibody for human VCAM-1 (Needham et al., 1994; Spertini et al., 1994), or mouse IgG1 isotype (clone 11711.11, R&D, Minneapolis, MN), at 0.05 μ g/ml for 5min, followed immediately by washing twice with TEM buffer and analysis in the parallel plate flow chamber.

For experiments in which leukocyte adhesion molecules were blocked, PBMC were incubated with recombinant humanized mouse anti-human $\alpha 4$ integrin IgG4k (natalizumab, a gift from Susan Goelz, Biogen Idec, Cambridge, MA), or human IgG4k isotype control (Sigma-Aldrich Inc. St. Louis, MO), at 0.05 μ g/ml for 10 min. Alternatively, PBMC were incubated with FN CS-1 peptide CYLHGPEILDVPST or control peptide (FN-CS1c) DELPQLVTLPHP (gifts from Dr. Mark Newman, Pharmexa-Epimmune Inc. San Diego, CA) at 1.875 μ M for 10 min, followed by perfusion across a THBMEC without washing.

2.4. Image analysis

Images captured during 20 min were made into a movie with QCapture software. The motions of each cell that flowed into the field and became adhesive during the shear stress application period was tracked and quantified. Adhesion includes the following three types of motion: Pure arrest was defined as cells that remained stationary throughout the period; Locomotion was defined as cells that spread and migrated over the endothelial surface without detaching or crossing the endothelial cell barriers, remaining phase bright; Transmigration was defined as cells that underwent phase change from bright to dark, indicating that they transmigrated through the endothelial cell barriers after spreading, migrating for variable distances on the endothelial surface.

2.5. Immunocytochemistry of tight junction associated protein

THBMEC were cultured on collagen-coated glass slides, followed by cytokine stimulation for 24 hours or PBS control treatment. THBMEC slides were assembled on the parallel plate flow chamber, and TEM was perfused across THBMEC at 9 ml/hr for 20 min. As a no shear-stress control, THBMEC slides were assembled on the parallel plate flow chamber for 20 min without turning on the flow. THBMEC were fixed by 4% PFA, and ZO-1, JAM-A, and VE-cadherin were detected by indirect immunocytochemistry. Rabbit anti-ZO-1 (Zymed Laboratories, San Francisco, CA), mouse anti-JAM-A antibodies (Clone J3F.1, Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-VE-cadherin (Clone F-8, Santa Cruz Biotechnology, Santa Cruz, CA) were used in combination with Alexa Fluor 594 goat anti-mouse IgG, Alexa Fluor 488 goat anti-rabbit IgG (H+L), and Alexa Fluor 488 goat anti-mouse IgG (Invitrogen Corporation, Carlsbad, CA). Slides were viewed using a Leica Aristoplan laser scanning confocal microscope (Leica Wetzlar, Heidelberg, Germany).

2.6. RT-PCR, subcloning and sequencing

Total RNA was obtained from THBMEC using Trizol (Invitrogen Corporation, Carlsbad, CA). Reverse transcription (RT) and polymerase chain reaction (PCR) were performed according to the manufacturer's instructions, using Roche first strand cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany). PCR was performed using Roche Taq DNA polymerase. RNA from HUVEC was used as a control. The primers set for the various conditions were: GAPDH: Forward 5'-GGTGGAGGTCGGA GTCAACG-3' and Reverse 5'-CAAAGTTGTCATGGATGACC-3'; FH-CS1: Forward 5'-TTCCCCAACTGGT AACCCTT-3' and Reverse 5'-TTTAAAGCCTGATTCAGAC AT TCG-3'; Fibronectin: Forward 5'-GTGCCACTTCCCCTTCCTAT-3' and Reverse 5'-ATCCCACTGATC TCCAATGC-3'. VCAM-1 Forward 5'-ATGACATGCTTGAGC CAGG-3' and reverse 5'-GTGTCTCCTTCTTTGACACT-3'. PCR product of FN-CS1 was subcloned with pCR II TOPO-TA expression vector (Invitrogen Corporation, Carlsbad, CA), and the resulting clone was proven by sequencing to have an insert that was 100% identical to the target gene.

2.7. Chemokine antibody arrays

We measured a total of 36 chemokines produced by a THBMEC, compared with resting THBMEC, using chemokine antibody arrays according to the manufacturer's instructions

(Raybiotech Inc. Norcross GA). Briefly, THBMEC were cultured and activated with TNF- α and IFN- γ for 24 hours as outlined above. After supernatant was harvested, THBMEC were washed, and protein was abstracted using cell lysis buffer provided by the kit. Total protein from cell lysate and supernatant was carefully quantified using Bio-Rad protein assay dye reagent (Life Science Research, Hercules, CA), and diluted into a working concentration of 1 mg/ml. Nitrocellulose membranes, each containing 36 different antibodies in duplicate spots, were blocked for 1 hour, and incubated with 1ml of each sample at 4°C overnight. Membranes were washed, and incubated with a cocktail of biotin-conjugated antibodies specific for the different proteins. Membranes were developed with streptavidin-conjugated peroxidase and ECL chemiluminescence reagent, and then exposed to Kodak® BioMax™ light film (Sigma-Aldrich Inc., St. Louis, MO). Samples were processed simultaneously to decrease observational error. Experiments were repeated on two different passages of THBMEC. Autoradiographic films were scanned and digitized spots were quantified with Image J software (BioDiscovery Inc.). Local background intensities were subtracted from each spot, and the average of the duplicate spots for each protein was normalized to the average of six positive controls on each membrane.

2.8. Statistical analysis

Each adherent PBMC in a 0.15 mm² field was tracked for 20 min during shear application manually by an investigator blinded to the experiment. Student's t-test analyses were performed on total adherent cell number to ascertain statistical significance, after the data were confirmed to be normally distributed using Kolmogorov-Smirnov test. A p-value <0.05 was used to establish significance.

3. Results

3.1. Development of an activated dynamic BBB model to study leukocyte-brain endothelial cell interaction under flow conditions

Intact morphology and tight junction integrity under physiological flow are essential for dynamic BBB models, where the intention is to study leukocyte transmigration. ZO-1 is critical for tight junction function, and is linked to the actin cytoskeleton (Luscinskas et al., 2002). JAM co-distributes with tight junction components at the apical region of the intercellular cleft and plays a role in regulating monocyte transmigration (Martin-Padura et al., 1998). Vascular Endothelium cadherin (VE-cadherin) is an important determinant of microvascular integrity and, together with catenins, forms the complex that mediates early-recognition between ECs. (Navarro, et al., 1998; Vorbrodt and Dobrogowska, 2004). THBMEC maintained cobblestone morphology and immunoreactivity for tight junction-associated proteins ZO-1, JAM-A and VE-cadherin after shear stress with or without cytokine exposure (Fig 1a). In the conditions evaluated, the continuous immunoreactivity of ZO-1, JAM-A and VE-cadherin along the intercellular junctions suggested that tight junctions were intact during shear stress application (Fig 1a). Cytokine exposure did exert biological effects. Among 36 chemokines we measured by chemokine antibody array, chemokines of the GRO family were up-regulated. Using a common antibody to GRO family members CXCL1, CXCL2, and CXCL3, modestly higher levels of GRO peptide were detected both in cell culture media and in cell lysates of aTHBMEC (Fig 1b).

3.2. Cytokine activation of THBMEC-enhanced PBMC adhesion

In order to explore the impact of THBMEC inflammation on leukocyte adhesion, we compared PBMC adhesion on activated versus resting THBMEC under flow conditions. In the *in vitro* dynamic BBB system, cytokine mediated activation of THBMEC enhanced PBMC adhesion (Fig. 2a, supplementary movie1 and 2).

3.3. Go/i receptor signal is essential for PBMC adhesion to inflammatory THBMEC

Leukocyte adhesion to endothelium under flow conditions is contingent on signaling via G α i-linked G protein coupled receptors (GPCRs). Pertussis toxin (PTX) almost completely blocked PBMC adhesion (Fig 2b). This observation suggests that GPCR signaling is involved in PBMC adhesion to brain endothelial cells in the present system.

3.4. NTZ infusion inhibited leukocyte adhesion on activated THBMEC under flow conditions

Natalizumab was designed to inhibit α 4 integrin-dependent leukocyte-endothelial interaction. IFN β -1a is also used for MS treatment and acts through as yet unknown mechanisms. We evaluated the effect of NTZ or IFN β -1a, using *ex vivo* PBMC from treated patients. PBMC adhesion to aTHBMEC was reduced by 85 percent in subjects receiving NTZ, compared to MS patients treated with IFN- β 1a (Fig 3a). For control experiments, we used PBMC from healthy subjects, exposed *in vitro* to NTZ or isotype control immunoglobulins (IgG4k). *In vitro* exposure of NTZ blocked PBMC adhesion on aTHBMEC, while nonspecific human IgG4k did not exert a significant effect (Fig 3b).

3.5. FN CS1 is the ligand on brain endothelial cells for alpha 4 integrin-mediated leukocyte adhesion

The relevant α 4 β 1 integrin ligand on brain endothelial cells has remained uncertain. THBMEC expressed FN-CS1 mRNA with no detectable VCAM-1, even though HUVEC expressed both FN-CS1 and VCAM-1 (Fig 4a). We then used FN-CS1 peptide to occupy the leukocyte α 4 integrin binding site, and found that FC-CS1 peptide blocked PBMC adhesion to aTHBMEC, compared to control peptide (Fig 4b). Anti-FN-CS1 antibodies were incubated with aTHBMEC, and blocked PBMC adhesion to aTHBMEC, while isotype IgM had no effect (Fig 4c). We used two different anti-VCAM-1 antibodies, neither of which affected PBMC adhesion (Fig. 4c). These results demonstrated that α 4 integrin/FN-CS1 mediated PBMC adhesion on brain endothelial cells.

4. Discussion

The initial objective of this study was to develop an *in vitro* inflammatory BBB model to visualize sequential leukocyte-brain endothelial interactions under physiological flow conditions. The leukocyte extravasation process, including apical chemokine deposits, integrin engagement, firm adhesion, locomotion, and transendothelial migration, are all dependent on hemodynamic shear, a fundamental physiological feature of all leukocyte-endothelial interactions. The present dynamic *in vitro* BBB model using parallel plate flow chamber and THBMEC allows us to visualize leukocyte-brain endothelial interactions under a flow rate simulating the velocity in brain capillaries (Hudetz et al., 1996). THBMEC maintained their morphology and the distribution of immunoreactivity for tight junction-associated proteins under flow conditions. These findings indicate that the leukocyte-endothelial interaction was not a passive process caused by mechanical damage to THBMEC.

To simulate inflammatory BBB, we stimulated THBMEC with TNF- α and IFN- γ at concentrations seen in patients with sepsis or systemic inflammatory response syndrome (Brunner et al., 2004; Collighan et al., 2004; Kabir et al., 2003; Watanabe et al., 2005). Cytokines did not cause observable changes in the distribution of immunoreactivity for tight junction-associated proteins. These observations are consistent with our prior report that aTHBMEC maintained a high transendothelial electrical resistance (TEER) and low solute permeability after cytokine exposure at these concentrations (Ubogu et al., 2006). As a positive control, we showed that these inflammatory cytokines increased expression of the chemokine GRO peptides CXCL1-3, indicating a biological response. Compared with resting THBMEC, activation of THBMEC with cytokines was required for robust adhesion of leukocytes under

flow conditions (Fig 2a). The relationship between the enhancement of adhesion and up-regulation of chemokine and adhesion molecules is under investigation. When G (i/o) signaling was blocked with PTX, PBMC adhesion was almost completely abrogated. This indicates that PBMC adhesion to inflammatory brain endothelial cells is GPCR-dependent.

The $\alpha 4$ integrins are constitutively expressed on lymphocytes, monocytes, and eosinophils (Hemler, 1990). The interaction between $\alpha 4 \beta 1$ integrin on leukocytes and its counter-receptors on the vascular endothelium plays a key role in MS (Rice et al., 2005; Von Andrian and Engelhardt, 2003). It is considered likely that $\alpha 4 \beta 1$ integrin mediates activated effector memory T cell and monocyte extravasation across the BBB into inflamed MS lesions (Ransohoff, 2007). NTZ demonstrated efficacy for treatment of MS in two large clinical trials (Polman et al., 2006; Rudick et al., 2006). Using the dynamic activated BBB model, we report that NTZ administration to MS patients significantly reduced PBMC adhesion to aTHBMEC under flow conditions. It is worth mentioning that both natalizumab and IFN β -1a treatment affect leukocyte composition in MS. Natalizumab releases leukocytes from the bone marrow and leads to an approximate doubling of T cells, B cells, pre-B cells, and monocytes in the peripheral blood (Krumbholz et al., 2008). In MS patients receiving IFN β -1a treatment, the numbers of CD3+, CD8+ T cells, natural killer (NK) T cells per mL blood were slightly lower than in healthy control subjects (Sellebjerg et al., 2005). The changes of leukocyte compositions are not likely to account for effect of these medications on PBMC adhesion under flow, as PBMC from patients receiving IFN β -1a showed adhesive properties equal to those of healthy controls, while NTZ changes the numbers of PBMC but does not drastically alter their relative proportions. Because standardized numbers of PBMC were used for each experiment, we consider it likely that the direct effects of NTZ on cells, rather than flux in cell populations, led to the results reported here.

The brain endothelial ligand for leukocyte $\alpha 4$ integrin has been unclear (Ransohoff, 2007). $\alpha 4$ integrin has at least two potential vascular ligands, VCAM-1 and FN-CS1 (Elices et al., 1990; Guan and Hynes, 1990). VCAM-1 is expressed by human aortic endothelial cells (Ganji et al., 2008) and HUVEC, forming transmigratory cups around lymphocytes during diapedesis across HUVEC monolayers (Carman and Springer, 2004; Miyake et al., 2008; Engelhardt and Wolburg, 2004). VCAM-1 was involved in leukocyte homing into lung, as well as melanoma and other tissue (Hallgren et al., 2007; Deem et al., 2007). Fibronectin is a high-molecular-weight glycoprotein found in plasma, at the cell surface, and in the extracellular matrix. It is involved in a variety of biological functions, such as cell attachment, spreading, and migration. Fibronectin can be alternatively spliced in at least three regions, generating multiple molecular moieties (Gutman and Kornblihtt, 1987; Ting et al., 2000; Wagner et al., 2000). In autopsy MS brain tissues, FN CS-1 was detected on astrocyte endfeet and astrocytes at the lesion edge (Van et al., 2005). In EAE mice, blocking $\alpha 4 \beta 1$ integrin with antibodies or peptides harboring the CS-1 sequence prevented cellular infiltration in the CNS parenchyma (Kent et al., 1995; Van der Laan et al., 2002). FN-CS1 is expressed on THBMEC at rest, with a significant increase in surface expression following cytokine activation (Ubogu et al., 2006). We confirmed that FN-CS1 mRNA was constitutively expressed by THBMEC. The absence of VCAM-1 expression by THBMEC is consistent with previous reports of lack of VCAM-1 immunoreactivity on brain endothelium in autopsy MS tissue sections (Kivisakk et al., 2003; Peterson et al., 2002). Our present and previous results demonstrated that among the two $\alpha 4$ integrin receptors, FN-CS1 is selectively found on THBMEC. When stimulated with higher concentrations of cytokines, THBMEC also exhibited VCAM-1 expression, consistent with previous reports by other groups, using 50–100 fold greater concentrations than those used here (Kallmann et al., 2000; Wong et al., 1999). Cytokine concentration may play a key role in inducing different ligands for $\alpha 4$ integrins.

In order to test the function of FN-CS1, FN-CS1 peptide was introduced to occupy the ligand binding site of leukocyte $\alpha 4$ integrin before the adhesion assay. The result showed that PBMC adhesion to aTHBMEC was abrogated. Function-neutralizing antibodies to FN-CS1 also blocked PBMC adhesion to aTHBMEC. It has been reported that Jurkat T cell adhesion to RA synovium was mediated by the interaction between VLA-4 and FN-CS1, but not VCAM-1 (Elices et al., 1994). Monocyte and T cell migration across *in vitro* BBB toward CCL5 was mediated by $\alpha 4\beta 1$ integrin and FN-CS1, not VCAM-1 (Ubogu et al., 2006). Collectively, these data suggest that FN-CS1, is the ligand for leukocyte $\alpha 4$ integrin on activated brain endothelial cells *in vitro*.

In conclusion, we describe a dynamic activated BBB model, combining inflammatory human cytokine exposed THBMEC and parallel plate flow chamber. We found that anti- $\alpha 4$ integrin treatment of MS patients inhibited PBMC adhesion to inflammatory THBMEC, as compared with MS controls that received IFN $\beta 1a$. We found that the ligand for leukocyte $\alpha 4$ integrin on brain endothelial cells is FN-CS1. In addition to $\alpha 4$ integrin, GPCR signaling is essential for leukocyte adhesion on aTHBMEC. This study provides a tool to study leukocyte-brain endothelial interactions, and contributes to our understanding of the effect of anti- $\alpha 4$ integrin antibody in MS treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Reference List

- Bechmann I, Galea I, Perry VH. What is the blood-brain barrier (not)? Trends Immunol 2007;28:5–11. [PubMed: 17140851]
- Brunner M, Krenn C, Roth G, Moser B, Dworschak M, Jensen-Jarolim E, Spittler A, Sautner T, Bonaros N, Wolner E, Boltz-Nitulescu G, Ankersmit HJ. Increased levels of soluble ST2 protein and IgG1 production in patients with sepsis and trauma. Intensive Care Med 2004;30:1468–1473. [PubMed: 14991091]
- Burns AR, Bowden RA, MacDonell SD, Walker DC, Odebunmi TO, Donnachie EM, Simon SI, Entman ML, Smith CW. Analysis of tight junctions during neutrophil transendothelial migration. J Cell Sci 2000;113 (Pt 1):45–57. [PubMed: 10591624]
- Callahan MK, Williams KA, Kivisakk P, Pearce D, Stins MF, Ransohoff RM. CXCR3 marks CD4+ memory T lymphocytes that are competent to migrate across a human brain microvascular endothelial cell layer. J Neuroimmunol 2004;153:150–157. [PubMed: 15265673]
- Carman CV, Springer TA. A transmigratory cup in leukocyte diapedesis both through individual vascular endothelial cells and between them. J Cell Biol 2004;167:377–388. [PubMed: 15504916]
- Chaudhuri A, Yang B, Gendelman HE, Persidsky Y, Kanmogne GD. STAT1 signaling modulates HIV-1-induced inflammatory responses and leukocyte transmigration across the blood-brain barrier. Blood 2008;111:2062–2072. [PubMed: 18003888]
- Collighan N, Giannoudis PV, Kourgeraki O, Perry SL, Guillou PJ, Bellamy MC. Interleukin 13 and inflammatory markers in human sepsis. Br J Surg 2004;91:762–768. [PubMed: 15164448]
- Danton GH, Dietrich WD. Inflammatory mechanisms after ischemia and stroke. J Neuropathol Exp Neurol 2003;62:127–136. [PubMed: 12578222]
- Deem TL, Badala-Valencia H, Cook-Mills JM. VCAM-1 activation of endothelial cell protein tyrosine phosphatase 1. B J Immunol 2007;178:3865–3873.

- Doring A, Wild M, Vestweber D, Deutsch U, Engelhardt B. E- and P-selectin are not required for the development of experimental autoimmune encephalomyelitis in C57BL/6 and SJL mice. *J Immunol* 2007;179:8470–8479. [PubMed: 18056394]
- Elices MJ, Osborn L, Takada Y, Crouse C, Luhowskyj S, Hemler ME, Lobb RR. VCAM-1 on activated endothelium interacts with the leukocyte integrin VLA-4 at a site distinct from the VLA-4/fibronectin binding site. *Cell* 1990;60:577–584. [PubMed: 1689216]
- Elices MJ, Tsai V, Strahl D, Goel AS, Tollefson V, Arrhenius T, Wayner EA, Gaeta FC, Fikes JD, Firestein GS. Expression and functional significance of alternatively spliced CS1 fibronectin in rheumatoid arthritis microvasculature. *J Clin Invest* 1994;93:405–416. [PubMed: 8282813]
- Engelhardt B, Ransohoff RM. The ins and outs of T-lymphocyte trafficking to the CNS: anatomical sites and molecular mechanisms. *Trends Immunol* 2005;26:485–495. [PubMed: 16039904]
- Engelhardt B, Wolburg H. Mini-review: Transendothelial migration of leukocytes: through the front door or around the side of the house? *Eur J Immunol* 2004;34:2955–2963. [PubMed: 15376193]
- Ganji SH, Qin S, Zhang L, Kamanna VS, Kashyap ML. Niacin inhibits vascular oxidative stress, redox-sensitive genes, and monocyte adhesion to human aortic endothelial cells. *Atherosclerosis* 2009;202:68–75. [PubMed: 18550065]
- Guan JL, Hynes RO. Lymphoid cells recognize an alternatively spliced segment of fibronectin via the integrin receptor alpha 4 beta 1. *Cell* 1990;60:53–61. [PubMed: 2295088]
- Gutman A, Kornblihtt AR. Identification of a third region of cell-specific alternative splicing in human fibronectin mRNA. *Proc Natl Acad Sci U S A* 1987;84:7179–7182. [PubMed: 3478690]
- Hallgren J, Jones TG, Abonia JP, Xing W, Humbles A, Austen KF, Gurish MF. Pulmonary CXCR2 regulates VCAM-1 and antigen-induced recruitment of mast cell progenitors. *Proc Natl Acad Sci U S A* 2007;104:20478–20483. [PubMed: 18077323]
- Hartmann TN, Grabovsky V, Pasvolsky R, Shulman Z, Buss EC, Spiegel A, Nagler A, Lapidot T, Thelen M, Alon R. A crosstalk between intracellular CXCR7 and CXCR4 involved in rapid CXCL12-triggered integrin activation but not in chemokine-triggered motility of human T lymphocytes and CD34+ cells. *J Leukoc Biol* 2008;84:1130–1140. [PubMed: 18653785]
- Hemler ME. VLA proteins in the integrin family: structures, functions, and their role on leukocytes. *Annu Rev Immunol* 1990;8:365–400. [PubMed: 2188667]
- Hudetz AG, Fehér G, Kampine JP. Heterogeneous autoregulation of cerebrocortical capillary flow: evidence for functional thoroughfare channels? *Microvasc Res* 1996;51:131–136. [PubMed: 8812768]
- Kabir K, Keller H, Grass G, Minor T, Stueber F, Schroeder S, Putensen C, Paul C, Burger C, Rangger C, Neville LF, Mathiak G. Cytokines and chemokines in serum and urine as early predictors to identify septic patients on intensive care unit. *Int J Mol Med* 2003;12:565–570. [PubMed: 12964035]
- Kallmann BA, Hummel V, Lindenlaub T, Ruprecht K, Toyka KV, Rieckmann P. Cytokine-induced modulation of cellular adhesion to human cerebral endothelial cells is mediated by soluble vascular cell adhesion molecule-1. *Brain* 2000;123 (Pt 4):687–697. [PubMed: 10734000]
- Kent SJ, Karlik SJ, Cannon C, Hines DK, Yednock TA, Fritz LC, Horner HC. A monoclonal antibody to alpha 4 integrin suppresses and reverses active experimental allergic encephalomyelitis. *J Neuroimmunol* 1995;58:1–10. [PubMed: 7730443]
- Kivisakk P, Mahad DJ, Callahan MK, Trebst C, Tucky B, Wei T, Wu L, Baekkevold ES, Lassmann H, Staugaitis SM, Campbell JJ, Ransohoff RM. Human cerebrospinal fluid central memory CD4+ T cells: evidence for trafficking through choroid plexus and meninges via P-selectin. *Proc Natl Acad Sci U S A* 2003;100:8389–8394. [PubMed: 12829791]
- Kraus J, Ling AK, Hamm S, Voigt K, Oschmann P, Engelhardt B. Interferon-beta stabilizes barrier characteristics of brain endothelial cells in vitro. *Ann Neurol* 2004;56:192–205. [PubMed: 15293271]
- Krumbholz M, Meinl I, Kümpfel T, Hohlfeld R, Meinl E. Natalizumab disproportionately increases circulating pre-B and B cells in multiple sclerosis. *Neurology* 2008;71:1350–1354. [PubMed: 18936427]
- Lossinsky AS, Shivers RR. Structural pathways for macromolecular and cellular transport across the blood-brain barrier during inflammatory conditions. *Review Histol Histopathol* 2004;19:535–564.
- Luscinskas FW, Ma S, Nusrat A, Parkos CA, Shaw SK. The role of endothelial cell lateral junctions during leukocyte trafficking. *Immunol Rev* 2002;186:57–67. [PubMed: 12234362]

- Luster AD, Alon R, Von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol* 2005;6:1182–1190. [PubMed: 16369557]
- Mahad D, Callahan MK, Williams KA, Ubogu EE, Kivisakk P, Tucky B, Kidd G, Kingsbury GA, Chang A, Fox RJ, Mack M, Sniderman MB, Ravid R, Staugaitis SM, Stins MF, Ransohoff RM. Modulating CCR2 and CCL2 at the blood-brain barrier: relevance for multiple sclerosis pathogenesis. *Brain* 2006;129:212–223. [PubMed: 16230319]
- Man S, Ubogu EE, Ransohoff RM. Inflammatory cell migration into the central nervous system: a few new twists on an old tale. *Brain Pathol* 2007;17:243–250. [PubMed: 17388955]
- Man S, Ubogu EE, Williams KA, Tucky B, Callahan MK, Ransohoff RM. Human brain microvascular endothelial cells and umbilical vein endothelial cells differentially facilitate leukocyte recruitment and utilize chemokines for T cell migration. *Clin Dev Immunol* 2008;2008:384982. [PubMed: 18320011]
- Martin-Padura I, Lostaglio S, Schneemann M, Williams L, Romano M, Fruscella P, Panzeri C, Stoppacciaro A, Ruco L, Villa A, Simmons D, Dejana E. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol* 1998;142:117–127. [PubMed: 9660867]
- McCandless EE, Piccio L, Woerner BM, Schmidt RE, Rubin JB, Cross AH, Klein RS. Pathological expression of CXCL12 at the blood-brain barrier correlates with severity of multiple sclerosis. *Am J Pathol* 2008;172:799–808. [PubMed: 18276777]
- Miyake Y, Mochizuki M, Ito C, Itoigawa M, Osawa T. Antioxidative pyranonigrins in rice mold starters and their suppressive effect on the expression of blood adhesion molecules. *Biosci Biotechnol Biochem* 2008;72:1580–1585. [PubMed: 18540108]
- Navarro P, Ruco L, Dejana E. Differential localization of VE- and N-cadherins in human endothelial cells: Ve-cadherin competes with N-cadherin for junctional localization. *J Cell Biol* 1998;140:1475–1484. [PubMed: 9508779]
- Needham LA, Van DS, Pigott R, Edwards RM, Shepherd M, Hemingway I, Jack L, Clements JM. Activation dependent and independent VLA-4 binding sites on vascular cell adhesion molecule-1. *Cell Adhes Commun* 1994;2:87–99. [PubMed: 7521763]
- Peterson JW, Bo L, Mork S, Chang A, Ransohoff RM, Trapp BD. VCAM-1-positive microglia target oligodendrocytes at the border of multiple sclerosis lesions. *J Neuropathol Exp Neurol* 2002;61:539–546. [PubMed: 12071637]
- Polman CH, O'Connor PW, Havrdova E, Hutchinson M, Kappos L, Miller DH, Phillips JT, Lublin FD, Giovannoni G, Wajgt A, Toal M, Lynn F, Panzara MA, Sandrock AW. A randomized, placebo-controlled trial of natalizumab for relapsing multiple sclerosis. *N Engl J Med* 2006;354:899–910. [PubMed: 16510744]
- Ransohoff RM. Natalizumab for multiple sclerosis. *N Engl J Med* 2007;356:2622–2629. [PubMed: 17582072]
- Ransohoff RM, Man S, Ubogu EE. “Doing the locomotion” with the multistep paradigm. *Blood* 2007;109:1342–1343.
- Rice GP, Hartung HP, Calabresi PA. Anti-alpha4 integrin therapy for multiple sclerosis: mechanisms and rationale. *Neurology* 2005;64:1336–1342. [PubMed: 15851719]
- Rudick RA, Stuart WH, Calabresi PA, Confavreux C, Galetta SL, Radue EW, Lublin FD, Weinstock-Guttman B, Wynn DR, Lynn F, Panzara MA, Sandrock AW. Natalizumab plus interferon beta-1a for relapsing multiple sclerosis. *N Engl J Med* 2006;354:911–923. [PubMed: 16510745]
- Schreiber TH, Shinder V, Cain DW, Alon R, Sackstein R. Shear flow-dependent integration of apical and subendothelial chemokines in T-cell transmigration: implications for locomotion and the multistep paradigm. *Blood* 2007;109:1381–1386. [PubMed: 17038526]
- Sellebjerg F, Ross C, Koch-Henriksen N, Sørensen PS, Frederiksen JL, Bendtzen K, Sørensen TL. CD26 + CD4 + T cell counts and attack risk in interferon-treated multiple sclerosis. *Mult Scler* 2005;11:641–645. [PubMed: 16320722]
- Shulman Z, Pasvolsky R, Woolf E, Grabovsky V, Feigelson SW, Erez N, Fukui Y, Alon R. DOCK2 regulates chemokine-triggered lateral lymphocyte motility but not transendothelial migration. *Blood* 2006;108:2150–2158. [PubMed: 16772603]

- Spertini F, Wang AV, Chatila T, Geha RS. Engagement of the common leukocyte antigen CD45 induces homotypic adhesion of activated human T cells. *J Immunol* 1994;153:1593–1602. [PubMed: 7913942]
- Stins MF, Gilles F, Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. *J Neuroimmunol* 1997;76:81–90. [PubMed: 9184636]
- Ting KM, Rothaupt D, McCormick TS, Hammerberg C, Chen G, Gilliam AC, Stevens S, Culp L, Cooper KD. Overexpression of the oncofetal Fn variant containing the EDA splice-in segment in the dermal-epidermal junction of psoriatic uninvolved skin. *J Invest Dermatol* 2000;114:706–711. [PubMed: 10733677]
- Ubogu EE, Callahan MK, Tucky BH, Ransohoff RM. Determinants of CCL5-driven mononuclear cell migration across the blood-brain barrier. Implications for therapeutically modulating neuroinflammation. *J Neuroimmunol* 2006;179:132–144. [PubMed: 16857269]
- Van der Laan LJ, Van der GA, Wauben MH, Ruuls SR, Dopp EA, De Groot CJ, Kuijpers TW, Elices MJ, Dijkstra CD. Beneficial effect of modified peptide inhibitor of alpha4 integrins on experimental allergic encephalomyelitis in Lewis rats. *J Neurosci Res* 2002;67:191–199. [PubMed: 11782963]
- Van HJ, Bo L, Vos CM, Virtanen I, De Vries HE. Basement membrane proteins in multiple sclerosis-associated inflammatory cuffs: potential role in influx and transport of leukocytes. *J Neuropathol Exp Neurol* 2005;64:722–729. [PubMed: 16106221]
- Von Andrian UH, Engelhardt B. Alpha4 integrins as therapeutic targets in autoimmune disease. *N Engl J Med* 2003;348:68–72. [PubMed: 12510047]
- Vorbrodt AW, Dobrogowska DH. Molecular anatomy of interendothelial junctions in human blood-brain barrier microvessels. *Folia Histochem Cytobiol* 2004;42:67–75. [PubMed: 15253128]
- Wagner C, Pioch M, Meyer C, Iking-Konert C, Andrassy K, Hansch GM. Differentiation of polymorphonuclear neutrophils in patients with systemic infections and chronic inflammatory diseases: evidence of prolonged life span and de novo synthesis of fibronectin. *J Mol Med* 2000;78:337–345. [PubMed: 11001531]
- Wang H, Sun J, Goldstein H. Human immunodeficiency virus type 1 infection increases the in vivo capacity of peripheral monocytes to cross the blood-brain barrier into the brain and the in vivo sensitivity of the blood-brain barrier to disruption by lipopolysaccharide. *J Virol* 2008;82:7591–7600. [PubMed: 18508884]
- Watanabe E, Hirasawa H, Oda S, Matsuda K, Hatano M, Tokuhisa T. Extremely high interleukin-6 blood levels and outcome in the critically ill are associated with tumor necrosis factor- and interleukin-1-related gene polymorphisms. *Crit Care Med* 2005;33:89–97. [PubMed: 15644653]
- Wong D, Prameya R, Dorovini-Zis K. In vitro adhesion and migration of T lymphocytes across monolayers of human brain microvessel endothelial cells: regulation by ICAM-1, VCAM-1, E-selectin and PECAM-1. *J Neuropathol Exp Neurol* 1999;58:138–152. [PubMed: 10029097]
- Woolf E, Grigorova I, Sagiv A, Grabovsky V, Feigelson SW, Shulman Z, Hartmann T, Sixt M, Cyster JG, Alon R. Lymph node chemokines promote sustained T lymphocyte motility without triggering stable integrin adhesiveness in the absence of shear forces. *Nat Immunol* 2007;8:1076–1085. [PubMed: 17721537]

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at

Fig 1.

Characterization of the inflammatory dynamic BBB model. A: Neither shear stress nor cytokines cause observable alterations of immunoreactivity for tight junction-associated protein ZO-1, JAM-A, and VE-cadherin. THBMEC culture were activated with TNF- α 10 u/ml and IFN- γ 20 u/ml or control media for 24 hours followed by assemble of the parallel plate flow chamber. TEM was perfused across THBMEC at 9ml/hr for 20 min. As control, THBMEC slides were assembled on the parallel plate flow chamber for 20 min without starting the flow. ZO-1, JAM-A and VE-Cadherin were detected by indirect immunocytochemistry. Scale: 50 μ m. B: Cytokine activation of THBMEC up-regulated GRO production. THBMEC were cultured and activated with TNF- α and IFN- γ for 24 hours as outlined above. Protein was

abstracted from THBMEC. Nitrocellulose membranes, containing antibodies in duplicate spots, were incubated with protein sample. Membranes were developed and exposed to Kodak® BioMax™ light film. Digitized spots were quantified with Image J software. Local background intensities were subtracted from each spot, and the average of the duplicate spots for each protein was normalized to the average of six positive controls on each membrane. rTHBMEC: resting THBMEC in which TNF- α and IFN- γ were omitted; aTHBMEC: activated THBMEC with TNF- α and IFN- γ .

A **B** **Fig. 2.**

Cytokine activation of THBMEC enhanced PBMC adhesion and GPCR receptor activation is essential for PBMC adhesion to inflammatory THBMEC. A: THBMEC were cultured on 35mm collagen-coated tissue culture dishes to subconfluence, followed by incubation with TNF- α 10 u/ml and IFN- γ 20 u/ml or control media for 24 hours. THBMEC cultures were assembled with a parallel plate flow chamber and mounted on the stage of a Leica inverted phase contrast microscope. PBMC were perfused across THBMEC at 9ml/hr by a Medfusion 3010a syringe pump. Cell interactions on a field of 0.15 mm² were captured 2 frames per second with a CCD camera during 20 minutes of shear application. Cell images were manually

quantified by analysis of the video (n=4). B: PBMC was treated by 100 ng/ml of pre-activated pertussis toxin (PTX) for 10 min followed by adhesion assay using aTHBMEC (n=4). * $p < 0.05$

Fig. 3.

Natalizumab inhibited PBMC adhesion to inflammatory THBMEC. A: Natalizumab infusion to MS patients inhibited their leukocyte adhesion on inflammatory THBMEC. Nine MS patients were treated with natalizumab for at least three courses (300mg IV). Four MS patients were treated with interferon β - a 30 mcg IM weekly for at least 12 months. PBMC were isolated and adhesion assay were performed using aTHBMEC. B: In vitro natalizumab, not control IgG4k, inhibited PBMC adhesion to inflammatory THBMEC. PBMC were incubated with natalizumab, or human IgG4k isotype control at 0.05 μ g/ml, or same volume of TEM buffer (Control), for 10min, followed by adhesion assay using aTHBMEC (n=4). * $p < 0.05$

Fig. 4. FN-CS1, not VCAM-1, is the ligand on brain endothelial cells for alpha 4 integrin-mediated leukocyte adhesion. A: FN-CS1 RNA, but not VCAM-1 RNA, was detected on THBMEC. Total RNA was obtained from THBMEC. HUVEC was used as a control. RT-PCR was performed, and PCR products were analyzed with 1.5% agarose gel. B: FN-CS1 peptide blocked PBMC adhesion to inflammatory THBMEC. PBMC from healthy control were incubated with FN-CS1 peptide (FN-CS1) or its control peptide (FN-CS1c) at 1.875 μ M, or the same volume of TEM buffer (Control), for 10 min, followed by adhesion assay using aTHBMEC (n=4). C: Anti-FN-CS1 Ab blocked PBMC adhesion to aTHBMEC, while anti-VCAM-1 Ab had no significant effect. Activated THBMEC were incubated with mouse anti-

human FN CS-1 IgM, mouse IgM Isotype, rat anti-mouse&human VCAM-1 IgG1, or rat IgG1 isotype, at 0.05µg/ml, or same volume of TEM buffer, for 5min, followed immediately by adhesion assay. n=3. * $p<0.05$.

Table 1**Information of patients and healthy control**

MS+IFN β 1a: MS patients were treated with interferon β -a 30 mcg IM weekly for at least 12 months. MS+Natalizumab: MS patients received at least three courses of natalizumab infusion (300mg IV), following the recommendations outlined in the US Food and Drug Administration-mandated Tysabri Outreach Unified Commitment to Health (TOUCH) program, and tested negative for anti-natalizumab antibodies. All patients gave written informed consent, and all study protocols were approved by CCF IRB.

	Healthy Control	MS+IFN β 1a	MS+Natalizumab
Total	10	4	9
Female:Male	7:3	2:2	6:3
Age (y/o)	30.4 $\pm$ 9.1	42.9 $\pm$ 10.2	39 $\pm$ 8.1