Regular Article*Highlighted Paper selected by Editor-in-Chief***Selective Protein Expression Changes of Leukocyte-Migration-Associated Cluster of Differentiation Antigens at the Blood–Brain Barrier in a Lipopolysaccharide-Induced Systemic Inflammation Mouse Model without Alteration of Transporters, Receptors or Tight Junction-Related Protein**Kazuki Sato, Masanori Tachikawa,^{*,†} Michitoshi Watanabe, Yasuo Uchida, and Tetsuya Terasaki*Division of Membrane Transport and Drug Targeting, Graduate School of Pharmaceutical Sciences, Tohoku University; 6–3 Aramaki, Aoba, Sendai 980–8578, Japan.*

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Leukocyte migration across the blood–brain barrier (BBB) is an important step in the progression of brain dysfunction in systemic inflammation. The purpose of this study was to identify the key regulatory molecule(s) at the BBB among the cluster of differentiation (CD) antigens involved in leukocyte migration in lipopolysaccharide (LPS)-induced systemic inflammation based on their absolute protein expressions. Here, we identified the absolute expression levels of 17 CD antigens in isolated brain capillaries (Bcap) of LPS-administered mice. Among them, the expression levels of CD54 and CD106 were dramatically increased in LPS-administered mice compared to the control by 6.21- and 3.67-fold, respectively. In peripheral blood mononuclear cells, the expression levels of CD11a/CD18, the counter-receptor for CD54, were similar to those of CD54 in Bcap of LPS-administered mice. On the other hand, the expression level of CD49d, part of CD29/CD49d complex, which is the counter-receptor for CD106, was under the limit of quantification. It is thus likely that CD54 at the BBB is predominantly involved in promoting leukocyte migration across the BBB in systemic inflammation. The expression levels of CD9, CD49c and CD97, which are thought to be involved in cell-to-cell interaction, were decreased by 40–60% in Bcap of LPS-administered mice. In contrast, the expression levels of 9 transporters, 2 receptors, and 1 tight junction-related protein in Bcap of LPS-administered mice were essentially unchanged compared to the control. These results suggest that enhancement of leukocyte migration in systemic inflammation involves dynamic changes of CD antigens without alterations of other major functional molecules.

Key words blood–brain barrier; leukocyte migration; quantitative protein atlas; systemic inflammation

INTRODUCTION

Leukocyte migration across the blood–brain barrier (BBB), which consists of brain capillary endothelial cells linked by complex tight junctions, is an important step in the progression of brain dysfunction during inflammatory diseases such as sepsis^{1,2)} and multiple sclerosis.³⁾ Accumulating evidence has shown that leukocyte migration involves cluster of differentiation (CD) antigens, such as integrins and selectins, which mediate the sequential processes of leukocytes rolling on endothelial cells, activation of leukocytes, leukocyte–endothelial cell adhesion and transmigration of leukocytes across endothelial cells.^{4,5)} Therefore, inhibition of the CD antigens involved in the leukocyte–endothelial cell interactions would be an effective strategy to prevent pathological progression mediated by leukocyte migration across the BBB. Systemic inflammation drives BBB changes *via* systemic-derived or brain-derived inflammatory mediators and induces recruitment of various types of leukocytes including lymphocytes, neutrophils and monocytes across the BBB.^{1,6–10)} On the other hand, multiple sclerosis is an autoimmune disease of the central nervous system in which regional inflammatory reactions

in the brain and spinal cord recruit leukocytes.^{3,11,12)} In animal experimental models such as sepsis and multiple sclerosis, leukocyte migration has been suppressed by inhibiting CD antigens on either the BBB side or the leukocyte side.^{4,9,13,14)} Consequently, CD antigens involved in leukocyte–endothelial interaction are considered to be promising druggable targets.

Natalizumab, a humanized monoclonal antibody directed against the cell adhesion molecule α 4-integrin (CD49d) on leukocyte plasma membrane, has the ability to inhibit the leukocyte migration in relapsing multiple sclerosis.¹⁵⁾ However, natalizumab is thought to decrease normal immune protection by suppressing leukocyte function, leading to severe adverse effects such as progressive multifocal leukoencephalopathy (PML).¹⁶⁾ Other monoclonal antibody drugs that target leukocyte membrane molecules, such as efalizumab and rituximab, also cause PML.¹⁶⁾ We hypothesized that these adverse effects might be avoided by selecting CD antigens on endothelial cells as molecular targets for inhibiting leukocyte migration.

As a first step in evaluating this strategy, it is necessary to establish criteria for selecting a suitable target molecule(s) among the CD antigens on endothelial cells. The best target would be a molecule that makes a major contribution to the functional enhancement of leukocyte–endothelial cell interactions. Previous reports have shown that the expression levels of multiple CD antigens such as CD62E/E-selectin,¹⁷⁾

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CD62P/P-selectin,^{17,18} CD54/intracellular adhesion molecule-1 (ICAM-1),^{9,19} CD106/vascular cell adhesion molecule 1 (VCAM-1)^{19,20} are increased in brain endothelial cells of a lipopolysaccharide (LPS)-induced systemic inflammation mouse model and/or experimental autoimmune encephalomyelitis (EAE) mouse, which is an animal model of multiple sclerosis. Thus, CD antigens that are abundantly expressed and/or highly induced in brain capillaries (Bcap) exposed to conditions favoring leukocyte migration such as systemic inflammation and multiple sclerosis could be promising inhibitory targets. In other words, measuring the absolute protein expression levels is useful to uncover intra-molecular and inter-molecular differences in the abundance of CD antigens in Bcap and/or leukocytes under normal and pathological conditions, and may be helpful for identifying optimum therapeutic options. However, the absolute levels of CD antigens in endothelial cells and leukocytes have not yet been determined or compared between normal and disease conditions.

We have previously established LC-MS/MS-based quantitative targeted absolute proteomics (QTAP) with *in silico* selection of the peptide sequences to be quantified, and this technology has enabled us to prepare quantitative protein atlases of membrane transporters, receptors and tight junction related-proteins at the normal BBB in mouse, rat, monkey, marmoset and human.^{21–25} Thus, the QTAP strategy should be effective for simultaneous quantification of CD antigen expression amounts at the BBB and on leukocytes. The purpose of this study was to identify key regulatory molecules of CD antigens at the BBB that are involved in leukocyte migration in LPS-induced systemic inflammation and multiple sclerosis by establishing a quantitative protein atlas.

MATERIALS AND METHODS

Animals Male ddY mice (11–12 weeks old) and female C57BL/6J mice (11–15 weeks old) were purchased from SLC Japan (Shizuoka, Japan). Up to 5 mice were housed together in a plastic cage with a stainless steel lid. The mice were maintained on a 12-h light/dark cycle in a temperature-controlled environment with free access to food and water. All experiments were approved by the Institutional Animal Care and Use Committee in Tohoku University, and were performed in accordance with the guidelines of Tohoku University.

LPS-Induced Systemic Inflammation and EAE Mouse Models Leukocyte migration across the BBB is a common neurological feature in septic systemic inflammation^{1,2,9} and multiple sclerosis.³ Clarifying the expression profiles of the BBB functional proteins involved in leukocyte migration is important to uncover both common and specific mechanisms of neurological disease progression and to identify potential therapeutic targets at the BBB. Therefore, we focused here on LPS-administered mice as a model of systemic inflammation and EAE mice as a model of multiple sclerosis. To prepare the systemic inflammation model, LPS (*Escherichia coli* 0127:B8; Sigma-Aldrich, St. Louis, MO, U.S.A.) dissolved in saline was intraperitoneally administered to male ddY mice (30 mg/kg weight) at 6 h prior to isolation of the Bcap. The same volume of saline was administered to control mice. EAE was induced in female C57BL/6J mice using a Hooke kitTM MOG_{35–55}/CFA Emulsion PTX and Hooke Control KitTM (Hooke Laboratories, Lawrence, MA, U.S.A.) according to the manufacturer's in-

structions. Behavior was evaluated at day 0 (day of immunization), and every 2 d after day 7 with the following symptom assignment scoring: 0: no disease, 1: weakness of tail tonus, 2: completely limp tail, 3: abnormal gait, 4: complete hind limb paralysis, 5: fore limb and hind limb paralysis, 6: moribund or dead (Supplementary Fig. 1). After the induction of EAE, the first symptoms appeared between days 9 and 11, reaching a peak at around day 17, and then continuing at the same level up to day 29 (Supplementary Fig. 1). Therefore, we studied EAE mice at 14 and 29 d after immunization as models of the acute/progressive and chronic phases, respectively. The average scores of acute and chronic EAE mice were 2.78 ± 0.15 and 4.00 ± 0.00 , respectively.

Isolation of Bcap Mouse Bcap were prepared by the nylon mesh method, as described previously.²⁴ Briefly, 9–10 mice were anesthetized by intraperitoneal injection of pentobarbital, and transcardially perfused with phosphate-buffered saline (PBS) to remove circulating blood cells. The cerebrums were homogenized with unrotated strokes in solution B (101 mM NaCl, 4.6 mM KCl, 5 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 15 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4). The homogenates were centrifuged ($1000 \times g$, 10 min, 4°C), and the pellet was resuspended in the same solution. An equal volume of 32% dextran (Serva Electrophoresis GmbH, Heidelberg, Germany) was added to the suspension, and the mixture was centrifuged ($4500 \times g$, 15 min, 4°C). The pellet was resuspended in solution A (solution B containing 25 mM NaHCO₃, 10 mM glucose, 1 mM pyruvate, and 5 g/L bovine serum albumin). The suspension was successively passed through 210 μ m nylon mesh, 85 μ m nylon mesh and 20 μ m nylon mesh. The Bcap were collected on the 20 μ m nylon mesh. The collected pellet was suspended in hypotonic buffer (10 mM NaCl, 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 7.4), and sonicated to prepare whole-tissue lysate of Bcap. Protein concentration was determined by the Lowry method using DC protein assay reagent (Bio-Rad, Hercules, CA, U.S.A.). Bcap samples were stored at -80°C .

Isolation of Peripheral Blood Mononuclear Cells (PBMCs) PBMCs were separated from ethylenediaminetetraacetic acid (EDTA)-treated whole blood collected from 20–21 mice by density gradient centrifugation with Lympholyte-Mammal (Cedarlane Laboratories, Burlington, Canada) according to the manufacturer's instructions. The PBMCs layer were collected and washed by PBS ($400 \times g$, 15 min, 4°C) then resuspended in ice-cold hypotonic buffer containing 1.25 mM phenylmethylsulfonyl fluoride, and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, U.S.A.), and lysed by nitrogen cavitation at 600 psi for 15 min at 4°C in a pressure vessel (Parr, Moline, IL, U.S.A.) to afford whole-cell lysate. This lysate was centrifuged ($10000 \times g$, 10 min, 4°C), and the supernatant was ultracentrifuged ($100000 \times g$, 60 min, 4°C). The resultant pellet was suspended in ice-cold suspension buffer (250 mM sucrose, 10 mM Tris-HCl, pH 7.4) to afford crude membrane fraction. The protein concentrations of the whole-cell lysate and crude membrane fraction were determined by the Lowry method using DC protein assay reagent. Samples were stored at -80°C .

Peptide Sample Preparation for LC-MS/MS Analysis (Proteins to Peptides) The whole-tissue lysate obtained from Bcap, the whole-cell lysate and crude membrane fraction

from PBMCs were subjected to lysyl endopeptidase (Lys-C) and trypsin digestion as described previously.²²⁾ Briefly, samples were dissolved in solubilizing buffer (7M guanidine hydrochloride, 10mM EDTA-Na, 0.5M Tris-HCl, pH 8.5) and the solubilized proteins were *S*-carboxymethylated. The alkylated proteins were precipitated with a mixture of methanol and chloroform, and the pellet was dissolved in 6M urea in 0.1M Tris-HCl (pH 8.5). The solution was diluted 5-fold with 0.1M Tris-HCl (pH 8.5) containing 0.05% Protease-Max surfactant (Promega, Madison, WI, U.S.A.) and treated with Lys-C (Wako Pure Chemical Industries, Ltd., Osaka, Japan; enzyme/substrate ratio of 1:100) at 30°C for 3h. The Lys-C-digested sample was treated with *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK)-treated trypsin (Promega; enzyme/substrate ratio of 1:100) at 37°C for 16h. The digested sample was desalted with GL-TipTM SDB and GL-TipTM GC (GL Sciences Inc., Tokyo, Japan). The desalted sample was taken up in a solution containing 80% acetonitrile and 0.1% trifluoroacetic acid, and evaporated in a centrifugal concentrator CC-105 (TOMY; Heat Low, 1h) under vacuum. The residue was dissolved in 0.1% formic acid in water.

Synthesis of Stable-Isotope-Labeled Internal Standard Proteins for the Protein Quantification of CD Antigens
To establish a QTAP-based simultaneous protein quantification system for mouse CD antigens (CD1-100), we used artificial internal standard proteins of CD-I and CD-II, in which the sequences were composed of tandemly connected target peptides of CD antigens. The target quantification peptides of each CD antigen protein were selected according to established *in silico* criteria^{21,22)} and connected tandemly. The unique reference peptides VIAPVLGR (Reference_VI, Ref_VI) and LFGPSIPLAR (Reference_LF, Ref_LF) were used for the determination of peptide amounts, and a HAT-tag was incorporated as carboxy-terminal (C-terminal) extension for protein purification. The amino acid sequences of CD-I and CD-II proteins are shown in Supplementary Figs.

2 and 3, respectively. The CD-I and CD-II protein-encoding cDNAs were chemically synthesized by Fasmac (Kanagawa, Japan), and inserted into the plasmid pET-17b (Novagen, Darmstadt, Germany). The protein expression vectors were transfected into *E. coli* (BL21-CodonPlus DE3 RIPL Competent Cells, Stratagene, La Jolla, CA, U.S.A.). *E. coli* cells were incubated in normal lactose broth culture medium for non-isotope-labeled protein (CD-I-St and CD-II-St) synthesis, or in the C.H.L. culture medium containing ¹⁵N-labeled amino acid mixture (Chlorella Industry, Tokyo, Japan) for stable isotope-labeled protein (CD-I-IS and CD-II-IS) synthesis. Protein expression was induced in the presence of 1mM isopropyl β -D-1-thiogalactopyranoside in the medium (30°C, 3h). The synthesized CD-I and CD-II proteins were purified on HisPurTM Cobalt Spin Columns (Thermo Fisher Scientific, IL, U.S.A.) according to the manufacturer's instructions, and dialyzed in PBS. The purified protein was solubilized with 6M urea in 0.1M Tris-HCl (pH 8.5), *S*-carboxymethylated, and digested with Lys-C and trypsin as described in the above section on peptide sample preparation. To determine the protein amounts of CD-I and CD-II, the peptide amounts of Ref_VI and Ref_LF were quantified by using chemically synthesized non-isotope-labeled standard peptides (VIAPVLGR and LFGPSIPLAR) and stable isotope-labeled standard peptides (VIAPVLGR* and LFGPSIPLAR*, where R* indicates labeling with ¹³C and ¹⁵N in Ref_VI and Ref_LF). Reference peptides were monitored with four sets of selected reaction monitoring/multiple reaction monitoring (SRM/MRM) transitions (see Supplementary Table 1). The averaged value of the Ref_VI and Ref_LF peptide amounts was used as the concentration of each target peptide in each standard protein.

Quantitative Targeted Absolute Quantification (QTAP)
QTAP analyses were performed according to our previous report.²²⁾ As shown in Supplementary Tables 1 and 2, the sequences of target peptides for CD antigens, transporters, receptors, a tight junction-related protein and other molecules

Table 1. Protein Expression Levels of CD Antigens and Marker Proteins in Bcap and PBMCs of LPS-Administered Mice

	Protein expression levels (fmol/ μ g protein)								
	Bcap			PBMCs			PBMCs		
	Control	LPS-administered	Ratio LPS/control	Whole-cell lysates		Ratio LPS/control	Crude membrane fractions		Ratio LPS/control
				Control	LPS-administered		Control	LPS-administered	
CD antigens									
CD11a	U.L.Q. (<0.497)	U.L.Q. (<0.574)	—	3.58 $\pm$ 0.14	2.04 $\pm$ 0.18	0.570	9.83 $\pm$ 0.51	17.2 $\pm$ 0.9	1.75
CD18	0.234 $\pm$ 0.071	0.282 $\pm$ 0.038	1.20	3.15 $\pm$ 0.15	2.59 $\pm$ 0.14	0.822	11.6 $\pm$ 0.2	15.4 $\pm$ 0.3	1.33
CD29	20.5 $\pm$ 0.2	22.9 $\pm$ 0.5	1.12	14.3 $\pm$ 0.6	9.63 $\pm$ 0.33	0.672	42.3 $\pm$ 1.5	64.0 $\pm$ 1.5	1.51
CD49d	0.403 $\pm$ 0.034	0.360 $\pm$ 0.034	0.893	U.L.Q. (<0.632)	U.L.Q. (<0.463)	—	0.561 $\pm$ 0.008	U.L.Q. (<0.561)	<0.999
CD54	1.06 $\pm$ 0.12	6.56 $\pm$ 0.16	6.21	U.L.Q. (<2.72)	U.L.Q. (<2.50)	—	U.L.Q. (<1.83)	1.84 $\pm$ 0.12	>1.00
CD62E	U.L.Q. (<0.357)	U.L.Q. (<0.372)	—	U.L.Q. (<2.02)	U.L.Q. (<2.25)	—	U.L.Q. (<0.590)	U.L.Q. (<0.687)	—
CD62P	U.L.Q. (<0.872)	U.L.Q. (<1.28)	—	N.D.	N.D.	—	U.L.Q. (<6.15)	U.L.Q. (<7.39)	—
CD102	4.09 $\pm$ 0.10	4.91 $\pm$ 0.14	1.20	U.L.Q. (<1.64)	U.L.Q. (<2.16)	—	1.06 $\pm$ 0.08	1.14 $\pm$ 0.05	1.07
CD106	0.503 $\pm$ 0.041	1.84 $\pm$ 0.09	3.67	U.L.Q. (<1.54)	U.L.Q. (<1.30)	—	U.L.Q. (<1.14)	U.L.Q. (<1.17)	—
CD162	U.L.Q. (<0.146)	U.L.Q. (<0.157)	—	U.L.Q. (<0.102)	U.L.Q. (<0.0615)	—	0.709 $\pm$ 0.270	0.493 $\pm$ 0.201	0.696
Marker proteins									
Na ⁺ /K ⁺ ATPase	26.4 $\pm$ 0.3	31.3 $\pm$ 0.4	1.18	1.81 $\pm$ 0.10	1.06 $\pm$ 0.10	0.588	5.36 $\pm$ 0.19	5.76 $\pm$ 0.19	1.07
γ -Gtp	2.93 $\pm$ 0.18	3.43 $\pm$ 0.22	1.17	U.L.Q. (<1.83)	U.L.Q. (<1.88)	—	U.L.Q. (<0.701)	U.L.Q. (<0.827)	—
CD41	U.L.Q. (<0.746)	U.L.Q. (<0.792)	—	71.3 $\pm$ 3.1	55.6 $\pm$ 2.5	0.780	245 $\pm$ 9	356 $\pm$ 14	1.46

Each quantitative value represents the mean $\pm$ standard error of the mean (S.E.M.) ($n = 3$ –16 transitions) obtained from three to four analyses of one whole-tissue lysate of pooled Bcap isolated from 10 mouse cerebrums, or one whole-cell lysate or crude membrane fraction of pooled PBMCs isolated from whole blood collected from 20–21 mice. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/ μ g protein). N.D., Not determined.

Table 2. Protein Expression Levels of CD Antigens and Marker Proteins in Bcap of EAE Mice

	Protein expression levels (fmol/ μ g protein)					
	Bcap		Ratio EAE/Control	Bcap		Ratio EAE/Control
	Control	EAE (acute phase)		Control	EAE (chronic phase)	
CD antigens						
CD11a	U.L.Q. (<1.32)	U.L.Q. (<1.24)	—	U.L.Q. (<0.581)	U.L.Q. (<0.640)	—
CD18	0.406 $\pm$ 0.029	0.652 $\pm$ 0.040	1.61	0.342 $\pm$ 0.058	0.577 $\pm$ 0.059	1.69
CD29	25.4 $\pm$ 0.6	22.4 $\pm$ 0.5	0.882	27.6 $\pm$ 0.4	38.9 $\pm$ 0.4	1.41
CD49d	0.489 $\pm$ 0.019	0.438 $\pm$ 0.021	0.897	0.381 $\pm$ 0.043	0.485 $\pm$ 0.050	1.27
CD54	2.83 $\pm$ 0.28	3.04 $\pm$ 0.28	1.07	2.32 $\pm$ 0.12	3.53 $\pm$ 0.16	1.52
CD62E	U.L.Q. (<0.179)	U.L.Q. (<0.153)	—	U.L.Q. (<0.388)	U.L.Q. (<0.429)	—
CD62P	U.L.Q. (<1.95)	U.L.Q. (<2.04)	—	U.L.Q. (<1.10)	U.L.Q. (<1.38)	—
CD102	4.96 $\pm$ 0.10	3.75 $\pm$ 0.10	0.756	5.55 $\pm$ 0.14	6.53 $\pm$ 0.19	1.18
CD106	1.01 $\pm$ 0.06	0.985 $\pm$ 0.075	0.975	0.687 $\pm$ 0.032	0.904 $\pm$ 0.043	1.32
CD162	U.L.Q. (<0.131)	U.L.Q. (<0.124)	—	U.L.Q. (<0.203)	U.L.Q. (<0.181)	—
Marker proteins						
Na ⁺ /K ⁺ ATPase	33.2 $\pm$ 1.0	27.5 $\pm$ 0.8	0.829	29.7 $\pm$ 0.5	37.4 $\pm$ 0.5	1.26
γ -Gtp	3.77 $\pm$ 0.09	3.06 $\pm$ 0.08	0.811	5.09 $\pm$ 0.17	5.58 $\pm$ 0.22	1.10
CD41	U.L.Q. (<0.237)	U.L.Q. (<0.218)	—	U.L.Q. (<0.814)	U.L.Q. (<0.871)	—

Each quantitative value represents the mean $\pm$ S.E.M. ($n=6-16$ transitions) obtained from three to four analyses of one whole-tissue lysate of pooled Bcap isolated from 9–10 mouse cerebrums. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/ μ g protein).

were selected based on the *in silico* criteria reported previously.^{21,22} The LC-MS/MS system consisted of a NanoLC-Ultra 2D plus system (Eksigent Technologies, Dublin, CA, U.S.A.), and a TripleTOF5600 equipped with a NanoSpray III ion source (AB SCIEX, Framingham, MA, U.S.A.). The NanoLC-Ultra 2D plus system consisted of an analytical column (75 μ m $\times$ 15 cm, ReproSil-Pur C18-AQ 3 μ m 120 Å, Eksigent Technologies) with or without a trap column (200 μ m $\times$ 6 mm ReproSil-Pur C18-AQ 3 μ m 120 Å, Eksigent Technologies). The flow rates of the trap column and the analytical column were 4 μ L/min (3 min run-time) and 300 nL/min, respectively. Mobile phases A and B consisted of 0.1% formic acid in water and 0.1% formic acid in acetonitrile, respectively. When the NanoLC-Ultra 2D plus system consisted of an analytical column with a trap column, the gradient system was as follows: 100% A:0% B (0–0.2 min), a linear gradient of 100% A:0% B to 60% A:40% B (0.2–40 min), increased to 0% A:100% B (40–41 min), maintained at 0% A:100% B (41–50 min), reduced to 100% A:0% B (50–50.1 min), and then maintained at 100% A:0% B (50.1–80 min). When the NanoLC-Ultra 2D plus system consisted of an analytical column without a trap column, the gradient system was as follows: 100% A:0% B (0–0.2 min), a linear gradient of 100% A:0% B to 50% A:50% B (0.2–50 min), increased to 0% A:100% B (50–51 min), maintained at 0% A:100% B (51–60 min), reduced to 100% A:0% B (60–60.1 min), and then maintained at 100% A:0% B (60.1–70 min). Other conditions were described previously.²⁶ The SRM/MS transitions of each peptide were set as shown in Supplementary Table 1 for QTAP with the chemically synthesized internal standard peptides and in Supplementary Table 2 for QTAP with the artificial internal standard proteins CD-I-IS and CD-II-IS. 1 μ g protein of each sample was subjected to LC-MS/MS analysis. For absolute quantification of the CD antigens using the CD-I and CD-II proteins, digested CD-I-IS or CD-II-IS protein-derived peptides equivalent to 100 fmol were spiked in the digested Bcap samples (1 μ g protein). The mixed samples were desalted with

GL-TipTM SDB and GL-TipTM GC (GL Sciences Inc., Tokyo, Japan). The samples were dissolved in 0.1% formic acid in water, and analyzed as described above. Calibration curves were prepared with various amounts (0–100 fmol) of peptides derived from CD-I-St and CD-II-St, and 100 fmol peptides derived from CD-I-IS and CD-II-IS. The protein expression levels were calculated as the average of 2–16 values obtained from two to four SRM/MS transitions in one to four analyses. A peak was defined as positive when the peak area was greater than 1000 counts, and the peaks derived from the different product ions were detected at the same retention times as those of the internal standard peptides. If no positive peaks were observed or a positive peak was detected in only one SRM/MS transition in one to four measurements, the protein expression level was defined to be under the limit of quantification (U.L.Q.). The value of the U.L.Q. was calculated according to our previous report.²⁷

RESULTS

QTAP Analysis of Adhesion Molecules with Chemically Synthesized Internal Standard Peptides in Bcap and/or PBMCs of LPS-Induced Systemic Inflammation and EAE Mouse Models Tables 1 and 2 summarize the protein expression levels of adhesion molecules of CD11a, CD18, CD29, CD49d, CD54, CD62E, CD62P, CD102, CD106, and CD162, which are well-established as leukocyte–brain endothelial cell interaction-related CD antigens,^{4,5} as well as several markers of Bcap and PBMCs of LPS-administered mice and the control (Table 1), and Bcap of EAE and control mice (Table 2). There were over 10-fold differences among the expression levels of CD18, CD29, CD49d, CD54, CD102 and CD106 in Bcap of LPS-administered mice and the control (Table 1 and Supplementary Fig. 4), while the levels of CD11a, CD62E, CD62P and CD162 were the U.L.Q. The expression levels of CD54 and CD106 were dramatically increased in Bcap of LPS-administered mice compared to the control mice by 6.21-

Table 3. Protein Expression Levels of CD Antigens in Bcap of LPS-Administered Mice Determined by Using CD-I-IS and CD-II-IS-Derived Internal Standard Peptides

	Protein expression levels (fmol/ μ g protein)		
	Control	LPS-administered	Ratio LPS/control
CD9	10.9 $\pm$ 0.1	5.79 $\pm$ 0.10	0.533
CD26	1.88 $\pm$ 0.03	1.66 $\pm$ 0.08	0.879
CD31	3.25 $\pm$ 0.16	3.11 $\pm$ 0.11	0.958
CD49a	9.56 $\pm$ 0.31	6.93 $\pm$ 0.15	0.725
CD49c	1.46 $\pm$ 0.22	0.862 $\pm$ 0.165	0.589
CD49f	5.92 $\pm$ 0.22	4.92 $\pm$ 0.14	0.831
CD71	6.68 $\pm$ 0.20	5.17 $\pm$ 0.07	0.773
CD82	0.913 $\pm$ 0.080	0.766 $\pm$ 0.229	0.839
CD90	4.52 $\pm$ 0.34	2.49 $\pm$ 0.16	0.550
CD97	0.436 $\pm$ 0.031	0.177 $\pm$ 0.074	0.407
CD98	13.0 $\pm$ 0.6	10.4 $\pm$ 0.4	0.804

Each quantitative value represents the mean $\pm$ S.E.M. ($n = 3-4$ transitions) in one analysis of one whole-tissue lysate of pooled Bcap isolated from 10 mouse cerebrums.

and 3.67-fold, respectively (Table 1 and Supplementary Fig. 4). In the whole-cell lysates and crude membrane fractions of PBMCs of LPS-administered mice and the control, the differences in the expression levels of CD11a, CD18, CD29, CD102, and CD162 were within 2-fold (Table 1). CD49d and CD54 were quantified only in crude membrane fraction of PBMCs of control and in LPS-administered mice, respectively (Table 1).

In contrast to the Bcap of LPS-administered mice, in the Bcap of EAE and control mice, the differences in expression levels of adhesion molecules of CD18, CD29, CD49d, CD54, CD102 and CD106 at the acute and chronic phases were all within 2-fold (Table 2).

QTAP Analysis with CD-I-IS and CD-II-IS-Derived Internal Standard Peptides in Bcap of LPS-Induced Systemic Inflammation Mice The use of biosynthesized artificial proteins composed of tandemly connected target peptides is a useful strategy for the quantitative analysis of multiple proteins by LC-MS/MS.²⁸⁾ To establish quantification systems for CD antigens, we synthesized non-isotope-labeled proteins (CD-I-St and CD-II-St) and stable isotope-labeled proteins (CD-I-IS and CD-II-IS). By using CD-I and CD-II standard proteins, we could establish quantification systems for 51 CD antigens (Supplementary Table 2), and firstly determined their expression levels in Bcap of control mice by QTAP. Among them, the expression levels of 11 CD antigens (CD9, CD26, CD31, CD49a, CD49c, CD49f, CD71, CD82, CD90, CD97, and CD98) were determined (Table 3), but the 40 CD antigens were under the limit of quantification (Table 4). Moreover, the expression levels of 11 CD antigens were also determined in Bcap of LPS-administered mice (Table 3). The comparative results showed the expression levels of CD9, CD49c, CD90 and CD97 in LPS-administered mice were decreased by 40–60% compared to those in control mice (Table 3 and Supplementary Fig. 4).

QTAP Analysis of Transporters, Receptors, and a Tight Junction-Related Protein in Bcap and/or PBMCs of LPS-Induced Systemic Inflammation and EAE Mouse Models To compare the changes in expression profiles between CD antigens and other transport systems, the protein amounts of transporters, receptors, and a tight junction-related protein

Table 4. List of CD Antigens with Expression Levels below the Limit of Quantification in Bcap of Normal Mice

Limit of quantification (fmol/ μ g protein)			
CD3e	1.78	CD42c	0.165
CD3g	2.70	CD42d	4.46
CD4	3.51	CD44	2.51
CD6	2.18	CD45	0.302
CD7	2.54	CD46	0.400
CD10	5.46	CD49b	0.940
CD11c	0.852	CD55	0.557
CDw12	1.44	CD64	1.61
CD14	3.66	CD68	0.586
CD19	1.62	CD69	1.03
CD20	0.560	CD72	0.801
CD21	0.882	CD73	2.25
CD27	1.06	CD74	0.166
CD30	1.12	CD79a	0.293
CD32	0.744	CD80	0.140
CD34	0.461	CD83	3.53
CD36	0.245	CD84	1.46
CD37	1.14	CD86	3.13
CD39	0.370	CD92	0.120
CD42b	0.467	CD100	0.166

The value represents the quantification limit for each protein in Bcap of normal mice (fmol/ μ g protein).

were determined in Bcap and PBMCs of LPS-administered mice and the control mice (Table 5), and in Bcap of EAE and control mice in the acute and chronic phases (Table 6). In Bcap of LPS-administered mice and the control mice, the differences were all less than 1.5-fold (Table 5 and Supplementary Fig. 5). In crude membrane fraction of PBMCs of LPS-administered mice and the control mice, the expression levels of Bcrp and Ent1 were respectively 2.24- and 2.74-fold greater in LPS-administered mice than in control mice, whereas the differences in the other quantified proteins were within 2-fold. In Bcap of EAE (in both the acute and chronic phases) and control mice, all of the quantified proteins showed less than 1.5-fold differences (Table 6).

DISCUSSION

The present study is the first to clarify the absolute protein expression levels of CD antigens in Bcap of LPS-induced systemic inflammation and EAE mouse models, as well as PBMCs of LPS-induced systemic inflammation mouse. Numerous studies have shown that CD antigens, including integrins, selectins, and cellular adhesion molecules (CAMs), are expressed in brain capillary endothelial cells and/or leukocytes.^{4,5)} We consider that selection of candidate therapeutic targets guided by knowledge of the absolute protein expression levels of these molecules under pathological conditions might be an effective approach to identify the most appropriate target(s) for inhibiting leukocyte migration at the BBB.

Our present results indicated that the absolute expression levels of CD54 and CD106, which are involved in leukocyte-endothelial cell interaction, were dramatically increased in Bcap of LPS-administered mice compared to the control mice, among CD antigens quantified in this study (Table 1). The increased protein expression levels of CD54 and CD106 in brain

Table 5. Protein Expression Levels of Transporters, Receptors and a Tight Junction-Related Protein in Bcap and PBMCs of LPS-Administered Mice

	Protein expression levels (fmol/μg protein)					
	Bcap		Ratio LPS/control	PBMCs (crude membrane fractions)		Ratio LPS/control
	Control	LPS-administered		Control	LPS-administered	
ABC transporters						
Abcb1a/Mdr1a	14.3 ± 0.2	16.8 ± 0.3	1.17	U.L.Q. (<0.526)	U.L.Q. (<0.578)	—
Abcb1b/Mdr1b	U.L.Q. (<1.59)	U.L.Q. (<1.98)	—	U.L.Q. (<1.91)	U.L.Q. (<2.77)	—
Abcb11/Bsep	U.L.Q. (<0.418)	U.L.Q. (<0.450)	—	U.L.Q. (<0.567)	U.L.Q. (<0.436)	—
Abcc4/Mrp4	0.827 ± 0.016	0.961 ± 0.031	1.16	U.L.Q. (<0.494)	U.L.Q. (<0.609)	—
Abcg2/Bcrp	4.02 ± 0.08	3.90 ± 0.09	0.970	0.767 ± 0.044	1.72 ± 0.05	2.24
SLC transporters						
Slc2a1/Glut1	123 ± 2	152 ± 3	1.23	0.505 ± 0.033	0.544 ± 0.066	1.08
Slc2a3/Glut3	0.460 ^{a)}	0.424 ^{a)}	0.921	15.5 ± 0.4	24.6 ± 0.6	1.59
Slc6a6/Taut	U.L.Q. (<1.44)	U.L.Q. (<1.42)	—	U.L.Q. (<1.35)	U.L.Q. (<1.53)	—
Slc16a1/Mct1	20.7 ± 0.5	23.0 ± 0.4	1.11	4.03 ± 0.35	7.18 ± 0.32	1.78
Slc22a8/Oat3	0.998 ± 0.06	0.805 ± 0.056	0.807	U.L.Q. (<0.770)	U.L.Q. (<0.894)	—
Slc27a1/Fatp1	1.47 ± 0.16	1.72 ± 0.27	1.17	U.L.Q. (<2.17)	U.L.Q. (<2.36)	—
Slc29a1/Ent1	0.548 ± 0.049	0.583 ± 0.058	1.06	0.489 ± 0.033	1.34 ± 0.04	2.74
Receptors						
Insr	0.567 ± 0.044	0.685 ± 0.054	1.21	0.450 ± 0.116	0.503 ^{a)}	1.12
Lrp1	0.531 ± 0.051	0.537 ± 0.038	1.01	U.L.Q. (<0.349)	U.L.Q. (<0.382)	—
Lrp2	U.L.Q. (<4.33)	U.L.Q. (<4.56)	—	U.L.Q. (<4.48)	U.L.Q. (<5.46)	—
Tight junction-related protein						
Claudin-5	3.30 ± 0.10	3.82 ± 0.11	1.16	U.L.Q. (<1.08)	U.L.Q. (<1.18)	—
Other						
Basigin	17.3 ± 0.4	18.3 ± 0.4	1.06	6.25 ± 0.23	8.20 ± 0.25	1.31

Each quantitative value represents the mean $\pm$ S.E.M. ($n=4-16$ transitions) obtained from three to four analyses of one whole-tissue lysate of pooled Bcap isolated from 10 mouse cerebrums, or one crude membrane fraction of pooled PBMCs isolated from whole blood collected from 20–21 mice. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/ μ g protein). *a)* The expression levels were calculated as the average of 2 quantitative values obtained from two SRM/MRM transitions in one analysis. Insr; insulin receptor.

Table 6. Protein Expression Levels of Transporters, Receptors and a Tight Junction-Related Protein in Bcap of EAE Mice

	Protein expression levels (fmol/μg protein)					
	Bcap		Ratio EAE/control	Bcap		Ratio EAE/control
	Control	EAE (acute phase)		Control	EAE (chronic phase)	
ABC transporters						
Abcb1a/Mdr1a	17.7 ± 0.5	14.4 ± 0.5	0.809	18.8 ± 0.5	22.4 ± 0.4	1.19
Abcb1b/Mdr1b	U.L.Q. (<0.572)	U.L.Q. (<0.563)	—	U.L.Q. (<0.811)	U.L.Q. (<0.783)	—
Abcb11/Bsep	U.L.Q. (<0.111)	U.L.Q. (<0.101)	—	U.L.Q. (<0.0901)	U.L.Q. (<0.0952)	—
Abcc4/Mrp4	1.38 ± 0.18	1.70 ± 0.18	1.24	1.90 ± 0.13	2.50 ± 0.22	1.31
Abcg2/Bcrp	4.34 ± 0.10	3.60 ± 0.07	0.830	5.19 ± 0.09	6.24 ± 0.11	1.20
SLC transporters						
Slc2a1/Glut1	160 ± 3	135 ± 3	0.847	146 ± 2	195 ± 4	1.34
Slc2a3/Glut3	U.L.Q. (<0.473)	U.L.Q. (<0.456)	—	U.L.Q. (<0.460)	U.L.Q. (<0.469)	—
Slc6a6/Taut	U.L.Q. (<1.59)	U.L.Q. (<0.707)	—	U.L.Q. (<0.0532)	U.L.Q. (<0.352)	—
Slc16a1/Mct1	14.0 ± 0.3	10.7 ± 0.2	0.764	13.5 ± 0.3	14.5 ± 0.3	1.07
Slc22a8/Oat3	0.745 ± 0.030	0.689 ± 0.020	0.925	1.03 ± 0.06	1.15 ± 0.04	1.12
Slc27a1/Fatp1	0.662 ± 0.071	0.532 ± 0.071	0.804	0.543 ± 0.051	0.749 ± 0.053	1.38
Slc29a1/Ent1	0.764 ± 0.047	0.735 ± 0.115	0.962	0.577 ± 0.024	0.762 ± 0.049	1.32
Receptors						
Insr	0.677 ± 0.035	0.610 ± 0.015	0.900	0.554 ± 0.021	0.689 ± 0.020	1.24
Lrp1	0.403 ± 0.021	0.414 ± 0.024	1.03	0.354 ± 0.023	0.492 ± 0.032	1.39
Lrp2	U.L.Q. (<1.42)	U.L.Q. (<1.47)	—	U.L.Q. (<0.967)	U.L.Q. (<0.877)	—
Tight junction-related protein						
Claudin-5	3.28 ± 0.15	3.91 ± 0.09	1.19	5.96 ± 0.15	7.64 ± 0.17	1.28
Other						
Basigin	14.1 ± 0.5	9.98 ± 0.32	0.709	11.0 ± 0.4	12.5 ± 0.5	1.14

Each value represents the mean $\pm$ S.E.M. ($n=6-16$ transitions) obtained from three or four analyses of one whole-tissue lysate of pooled Bcap isolated from 9–10 mouse cerebrums. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/ μ g protein). Insr; insulin receptor.

endothelial cells in LPS-induced systemic inflammation are consistent with previous immunohistochemical results.^{20,29} It has been proposed that LPS and/or LPS-induced inflammatory cytokine (e.g. tumor necrosis factor- α (TNF- α)) increase the expression of CD54 and CD106 *via* activation of the transcription factors nuclear factor-kappaB (NF- κ B) and activator protein 1 (AP-1) in human endothelium.^{30–34} Similar mechanisms could lead to the upregulation of CD54 and CD106 in brain capillary endothelial cells of LPS-treated mice. On the other hand, the expression levels of transporters, receptors and a tight junction-related protein in Bcap of LPS-administered mice were essentially unchanged compared to the control. These results imply that leukocyte migration across the BBB in LPS-induced systemic inflammation is mediated predominantly by increased expression of migration-associated CD antigens, without dramatic alterations of other basic functional molecules at the BBB. CD54 and CD106 on brain capillary endothelial cells interact with lymphocyte function-associated antigen 1 (LFA-1; CD11a/CD18 complex) and very late antigen-4 (VLA-4; CD29/CD49d complex) on leukocytes during the adhesion process.⁵ Our present data indicate that the expression levels of both CD11a and CD18 in PBMCs (whole-cell lysates) are similar to that of CD54 in Bcap (about 3-fold difference) in LPS-administrated mice (Table 1). In contrast, the expression level of CD49d in PBMCs (whole-cell lysate) was U.L.Q. in both LPS-administered mice and the control; further that in crude membrane fraction of normal PBMCs was 0.561 fmol/ μ g protein, whereas that in PBMCs of LPS-administered mice was under the quantification limit (U.L.Q. < 0.561 fmol/ μ g protein). These results suggest that the leukocyte-endothelial cell binding of CD29/CD49d complex with CD106 at the BBB of LPS-administered mice is unchanged or even suppressed, compared with control mice. Thus, it appears that inhibition of the binding of CD54 with CD11a/CD18 would be more effective to prevent leukocyte migration across the BBB. In support of this notion, previous studies have shown that lymphocyte adhesion to, and/or transmigration across, inflammatory-cytokine-activated brain capillary endothelial cells were significantly inhibited by depletion of endothelial CD54,³⁵ anti-CD54,^{35,36} as well as by single treatment with anti-LFA-1 (CD11a/CD18 complex)³⁷ antibodies, whereas anti-CD106³⁶ and anti-VLA-4 (CD29/CD49d complex)³⁷ antibodies had modest effect. Furthermore, anti-CD54 antibody reduced neutrophil accumulation in lung of cecal ligation puncture (CLP)-septic systemic inflammation mice, but anti-CD106 antibody or anti-VLA-4 (CD29/CD49d complex) antibody had no effect.³⁸ Interestingly, CD54-depleted mice showed not only reduced cell migration into the brain,⁹ but also reduced mortality³⁹ in LPS-induced systemic inflammation. These lines of evidence strongly suggest that CD54 is a promising target at the BBB for inhibition of leukocyte migration in systemic inflammation.

Activation of integrins such as CD11a and CD18 on leukocytes is a crucial step of leukocyte migration across the BBB.³⁷ However, our present results indicate that the protein expression levels of CD11a and CD18 in PBMCs of LPS-administered mice were essentially unchanged compared to the control. The activation of integrins could be explained by the fact that the binding affinities of integrins are increased by conformation changes and clustering.⁴⁰ On the other hand, it has been reported that the activation of CAMs such as

CD54 and CD106 involves increased expression levels.^{9,19,20,41} From the viewpoint of the absolute protein levels obtained in the present study, the expression of CD54 (1.06 fmol/ μ g protein) in Bcap is less than that of CD11a/CD18 (3.58 and 3.15 fmol/ μ g protein, respectively) in PBMCs under normal conditions, whereas the expression of CD54 (6.56 fmol/ μ g protein) exceeds that of CD11a/CD18 (2.04 and 2.59 fmol/ μ g protein, respectively) in LPS-administered mice. It is thus possible that leukocyte migration across the BBB is due at least in part to increased binding affinities of CD11a and CD18 in PBMCs, as well as the increased maximum binding capacity of CD54 due to the increased protein levels in Bcap.

QTAP with the CD-I-IS and CD-II-IS internal standard proteins allowed us to determine the expression levels of 11 CD antigens in Bcap of LPS-administered mice and the control (Table 3). Among the detected CD antigens, the expressions of CD9,⁴² CD26,⁴³ CD31,⁴⁴ CD49a,⁴⁵ CD49c,⁴⁵ CD49f,⁴⁵ CD71,⁴⁶ CD82,⁴⁷ CD97⁴⁷ and CD98⁴⁸ at the BBB are consistent with previous findings. Although protein expression of CD90, which is predominantly expressed in brain pericytes,⁴⁹ was detected in Bcap, it is presumably derived from brain capillary-contacting pericytes. The present results indicate that the expression levels of CD9, CD49c and CD97 are decreased by 40–60% in Bcap of LPS-administered mice (Table 3). An immunoprecipitation study has demonstrated that CD9 and CD49c would form a complex at the cell-to-cell junctions of human umbilical vein endothelial cell (HUVEC).⁵⁰ Furthermore, the direct interaction of CD9 with a tight junction-related protein claudin-1 was demonstrated by means of a cross-linking proteomics study.⁵¹ We have recently reported that claudin-5 and claudin-11 are expressed at the human and rodent BBB.⁵² Although it is unclear whether CD9 interacts with claudin-5 or claudin-11 at the BBB, these results imply that reduced expression of CD9 may affect the integrity of endothelial cell-to-cell junctions. The function of CD97 in BBB is still unknown, but CD97 is also expressed preferentially in E-cadherin-based adherens junctions of colonic enterocytes, and CD97 overexpression increased the structural integrity of adhesion junctions of enterocytes by promoting lateral cell-to-cell interactions, which, in contrast, were weakened in CD97 knockout mice.⁵³ Leukocyte migration across the BBB is considered to occur *via* not only a transcellular route, but also a paracellular route.^{54,55} These lines of evidence suggest that the decreased levels of CD9, CD49c and CD97 are involved in the enhancement of leukocyte migration *via* the paracellular route across the BBB in LPS-administered mice by affecting endothelial cell-to-cell interaction at the BBB.

The absolute protein expression levels determined by using CD-I-IS and CD-II-IS proteins as internal standards could be affected by incomplete trypsin digestion. However, in this study, the expression levels of CD71 (transferrin receptor 1, 6.68 fmol/ μ g protein) and CD98 (4F2hc, 13.0 fmol/ μ g protein) in Bcap of control ddY mouse determined by using CD-I and CD-II standard proteins were consistent with reported expression levels of CD71 (4.34–5.84 fmol/ μ g protein)^{22,56} and CD98 (16.4–17.2 fmol/ μ g protein),^{21,56} determined by using chemically synthesized internal standard peptides. Thus, we think that the standard proteins would have been almost completely digested by trypsin under the conditions of the present study.

In Bcap derived from the cerebrum of the EAE model mice (in both the acute and chronic phases) and control mice, we

did not find any dramatic difference (*i.e.*, less than 2-fold) in CD antigens, transporters, receptors or a tight junction-related protein. This could be explained by the fact that the site of disease onset in the EAE mouse brain is localized,¹²⁾ and our analysis of Bcap isolated from whole cerebrum would not have detected such local protein expression changes. In support of this notion, previous reports have demonstrated that (i) the first entry of leukocytes into the central nervous system (CNS) occurs at the dorsal blood vessels of the fifth lumbar segment of the spinal cord⁵⁷⁾ and (ii) CD54 is expressed in localized regions of EAE mouse brain.⁵⁸⁾ It would be intriguing in future studies to determine the absolute protein expression levels of CD antigens in the Bcap of restricted brain regions and the spinal cord capillaries of EAE mice, in which the leukocyte migration occurred.

In conclusion, our measurements here of the absolute protein expression levels of CD antigens in Bcap and leukocytes suggest that CD54 at the BBB is the most promising candidate as an inhibitory target molecule for the inhibition of leukocyte-migration in systemic inflammation. Moreover, we observed decreased expression of CD9, CD49c and CD97, which are thought to be involved in cell-to-cell interaction, suggesting their involvement in leukocyte migration as well. These findings should be helpful in the development of BBB-targeting drugs to block leukocyte migration associated with CNS disorders.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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