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

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INTRODUCTION

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 CD94d, 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

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CD62P/P-selectin, CD54/intracellular adhesion molecule-1 (ICAM-1), CD106/vascular cell adhesion molecule 1 (VCAM-1) 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 therapeutic 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. 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 and multiple sclerosis. 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 0127:8; 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 kit™ MOG35-55/CFA Emulsion PTX and Hooke Control Kit™ (Hooke Laboratories, Lawrence, MA, U.S.A.) according to the manufacturer’s instructions. 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. 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 cerebri 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)pyperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4). The homogenates were centrifuged (1000 × g, 10 min, 4°C), and the pellet was resuspended in the same solution. An equal volume of 32% dextran (Sera Electrophoresis GmbH, Heidelberg, Germany) was added to the suspension, and the mixture was centrifuged (4500 × 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 µm nylon mesh, 85 µm nylon mesh and 20 µm nylon mesh. The Bcap were collected on the 20 µm 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 Lymphocyte-Mammal (Cedarlane Laboratories, Burlington, Canada) according to the manufacturer’s instructions. The PBMCs layer were collected and washed by PBS (400 × 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 × g, 10 min, 4°C), and the supernatant was ultracentrifuged (100000 × 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...
CD-I and CD-II proteins are shown in Supplementary Figs. for protein purification. The amino acid sequences of was incorporated as carboxy-terminal (C-terminal) extension for the determination of peptide amounts, and a HAT-tag report. As shown in Supplementary Tables 1 and 2, the QTAP analyses were performed according to our previous criteria and connected tandemly. The unique reference peptides VIAPVLGR (Reference_VI, Ref_VI) and LFGPSIPLAR (Reference_LF, Ref_LF) were quantified by using chemically synthesized non-isotope-labeled standard peptides (VIAPVGLGR and LFGPSIPLAR) and stable isotope-labeled standard peptides (VIAPVGLGR* 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. 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

| CD antigens | Bcap | Ratio | PBMCs | Ratio | PBMCs | Ratio |
|-------------|------|-------|--------|-------|-------|-------|
|             | Control | LPS-administered | LPS/control | Whole-cell lysates | Crude membrane fractions | Control | LPS-administered |
| CD1a | U.L.Q. (<0.497) | U.L.Q. (<0.574) | — | 3.58 ± 0.14 | 2.04 ± 0.18 | 0.570 | 9.83 ± 0.51 | 17.2 ± 0.9 | 1.75 |
| CD18 | 0.234 ± 0.071 | 0.282 ± 0.038 | 1.20 | 3.15 ± 0.15 | 2.59 ± 0.14 | 0.822 | 11.6 ± 0.2 | 15.4 ± 0.3 | 1.33 |
| CD29 | 20.5 ± 0.2 | 22.9 ± 0.5 | 1.12 | 14.3 ± 0.6 | 9.63 ± 0.33 | 0.672 | 42.3 ± 1.5 | 64.0 ± 1.5 | 1.51 |
| CD49d | 0.403 ± 0.034 | 0.360 ± 0.034 | 0.893 | U.L.Q. (<0.632) | U.L.Q. (<0.463) | — | 0.561 ± 0.008 | U.L.Q. (<0.561) | <0.999 |
| CD54 | 1.06 ± 0.12 | 6.56 ± 0.16 | 6.21 | U.L.Q. (<2.72) | U.L.Q. (<2.50) | — | U.L.Q. (<1.83) | 1.84 ± 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 | 0.49 ± 0.10 | 4.91 ± 0.14 | 1.20 | U.L.Q. (<1.64) | U.L.Q. (<2.16) | — | 1.06 ± 0.08 | 1.14 ± 0.05 | 1.07 |
| CD106 | 0.503 ± 0.041 | 1.84 ± 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 ± 0.270 | 0.493 ± 0.201 | 0.696 |

| Markers | Ratio |
|---------|-------|
| Na⁺/K⁺ ATPase | 26.4 ± 0.3 | 31.3 ± 0.4 | 1.18 | 1.81 ± 0.10 | 1.06 ± 0.10 | 0.588 | 5.36 ± 0.19 | 5.76 ± 0.19 | 1.07 |
| γ-GTP | 2.93 ± 0.746 | 3.43 ± 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 ± 3.1 | 55.6 ± 2.5 | 0.780 | 245 ± 9 | 356 ± 14 | 1.46 |

Each quantitative value represents the mean ± 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/µg protein). N.D., Not determined.
were selected based on the *in silico* criteria reported previously.\textsuperscript{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 \( \mu \)m × 15 cm, ReproSil-Pur C18-AQ 3 \( \mu \)m 120 Å, Eksigent Technologies) with or without a trap column (200 \( \mu \)m × 6 mm ReproSil-Pur C18-AQ 3 \( \mu \)m 120 Å, Eksigent Technologies). The flow rates of the trap column and the analytical column were 4 \( \mu \)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.\textsuperscript{26} The SRM/MRM 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 \( \mu \)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 \( \mu \)g protein). The mixed samples were desalted with GL-Tip\textsuperscript{TM} SDB and GL-Tip\textsuperscript{TM} 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/MRM 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/MRM 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.\textsuperscript{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,\textsuperscript{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, CD62E, CD62P, CD102, CD106, and CD162 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-

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

| CD antigens | Protein expression levels (fmol/\( \mu \)g protein) |
|-------------|--------------------------------------------------|
|             | Bcap                                             | Control |
|             | EAE (acute phase)                                | Ratio   |
|             | EAE/Control                                      |         |
|             | Bcap                                             | Control |
|             | EAE (chronic phase)                              | Ratio   |
|             | EAE/Control                                      |         |
| CD11a       | U.L.Q. (<1.32)                                   | 1.61    |
| CD18        | 0.406 ± 0.029                                    |         |
| CD29        | 25.4 ± 0.6                                       | 0.882   |
| CD40d       | 0.489 ± 0.019                                    | 1.07    |
| CD54        | 2.83 ± 0.28                                      |         |
| CD62E       | U.L.Q. (<0.179)                                  | 1.07    |
| CD62P       | U.L.Q. (<1.95)                                   | 0.19    |
| CD102       | 4.96 ± 0.10                                      | 0.756   |
| CD106       | 1.01 ± 0.06                                      | 0.975   |
| CD162       | U.L.Q. (<0.131)                                  | 0.829   |
| Na\textsuperscript{+}/K\textsuperscript{+} ATPase| 33.2 ± 1.0                                      |         |
| \( \gamma \)-GTP | 3.77 ± 0.09                                   | 0.811   |
| CD41        | U.L.Q. (<0.237)                                  | 0.829   |

Each quantitative value represents the mean ± 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 cerebri. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/\( \mu \)g protein).
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 CD162 were within 2-fold (Table 1). CD49d and CD54 were increased in Bcap of EAE (in both the acute and chronic phases) and control mice in the acute and chronic phases (Table 6). In Bcap 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 Bcap of LPS-administered mice than in control mice, whereas 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 PBMCs of control 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

| Transports/Proteins | Bcap (crude membrane fractions) | PBMCs (crude membrane fractions) | Ratio LPS/control |
|---------------------|---------------------------------|-----------------------------------|-------------------|
| **ABC transporters**|                                 |                                   |                   |
| Abcb1a/Mdr1a        | 14.3 ± 0.2                      | 16.8 ± 0.3                        | 1.17              |
| Abcb1b/Mdr1b        | U.L.Q. (<1.59)                  | U.L.Q. (<1.98)                    | —                 |
| Abcb11/Bsep         | U.L.Q. (<0.418)                 | U.L.Q. (<0.450)                   | —                 |
| Abcc4/Mrp4          | 0.827 ± 0.016                   | 0.961 ± 0.031                     | 1.16              |
| Abcg2/Bcrp          | 4.02 ± 0.08                     | 3.90 ± 0.09                       | 0.970             |
| **SLC transporters**|                                 |                                   |                   |
| Slc2a1/Glut1        | 123 ± 2                         | 152 ± 3                           | 1.23              |
| Slc2a3/Glut3        | 0.460 (±0.044)                  | 0.424 (±0.052)                    | 0.921             |
| Slc6a9/Taut         | U.L.Q. (<1.444)                 | U.L.Q. (<1.42)                    | —                 |
| Slc16a1/Mct1        | 20.7 ± 0.5                      | 23.0 ± 0.4                        | 1.11              |
| Slc22a8/Oat3        | 0.998 ± 0.06                    | 0.805 ± 0.056                     | 1.26              |
| Slc2a1/Fatp1        | 1.47 ± 0.16                     | 1.72 ± 0.27                       | 1.17              |
| Slc2a9a1/Ent1       | 0.548 ± 0.049                   | 0.583 ± 0.058                     | 1.06              |
| **Receptors**       |                                 |                                   |                   |
| Insr                | 0.567 ± 0.044                   | 0.685 ± 0.054                     | 1.21              |
| Lrp1                | 0.531 ± 0.051                   | 0.537 ± 0.038                     | 1.01              |
| Lrp2                | U.L.Q. (<4.33)                  | U.L.Q. (<4.56)                    | —                 |
| **Tight junction-related protein** |                           |                                   |                   |
| Claudin-5           | 3.30 ± 0.10                     | 3.82 ± 0.11                       | 1.16              |
| **Other**           |                                 |                                   |                   |
| Basigin             | 17.3 ± 0.4                      | 18.3 ± 0.4                        | 1.06              |

Each quantitative value represents the mean ± 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 cerebroms, 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/µg 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

| Transports/Proteins | Bcap (acute phase) | Bcap (chronic phase) | Ratio EAE/control |
|---------------------|-------------------|----------------------|-------------------|
| **ABC transporters**|                   |                      |                   |
| Abcb1a/Mdr1a        | 17.7 ± 0.5        | 14.4 ± 0.5           | 0.809             |
| Abcb1b/Mdr1b        | U.L.Q. (<0.572)   | U.L.Q. (<0.563)      | —                 |
| Abcb11/Bsep         | U.L.Q. (<0.111)   | U.L.Q. (<0.101)      | —                 |
| Abcc4/Mrp4          | 1.38 ± 0.18       | 1.70 ± 0.18          | 1.24              |
| Abcg2/Bcrp          | 4.34 ± 0.10       | 3.60 ± 0.07          | 0.830             |
| **SLC transporters**|                   |                      |                   |
| Slc2a1/Glut1        | 160 ± 3           | 135 ± 3              | 0.847             |
| Slc2a3/Glut3        | U.L.Q. (<0.473)   | U.L.Q. (<0.456)      | —                 |
| Slc6a9/Taut         | U.L.Q. (<1.59)    | U.L.Q. (<0.707)      | —                 |
| Slc16a1/Mct1        | 14.0 ± 0.3        | 10.7 ± 0.2           | 0.764             |
| Slc22a8/Oat3        | 0.74 ± 0.030      | 0.689 ± 0.020        | 0.925             |
| Slc2a1/Fatp1        | 0.662 ± 0.071     | 0.532 ± 0.071        | 0.804             |
| Slc2a9a1/Ent1       | 0.764 ± 0.047     | 0.735 ± 0.115        | 0.962             |
| **Receptors**       |                   |                      |                   |
| Insr                | 0.677 ± 0.035     | 0.610 ± 0.015        | 0.900             |
| Lrp1                | 0.403 ± 0.021     | 0.414 ± 0.024        | 1.03              |
| Lrp2                | U.L.Q. (<1.42)    | U.L.Q. (<1.47)       | —                 |
| **Tight junction-related protein** |           |                      |                   |
| Claudin-5           | 3.28 ± 0.15       | 3.91 ± 0.09          | 1.19              |
| **Other**           |                   |                      |                   |
| Basigin             | 14.1 ± 0.5        | 9.98 ± 0.32          | 0.709             |

Each value represents the mean ± 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 cerebroms. U.L.Q., Under the limit of quantification. The values in brackets represent the quantification limits (fmol/µg protein). Insr; insulin receptor.
endothelial cells in LPS-induced systemic inflammation are consistent with previous immunohistochemical results.\textsuperscript{20,29} It has been proposed that LPS and/or LPS-induced inflammatory cytokine (e.g. tumor necrosis factor-alpha (TNF-\(\alpha\))) increase the expression of CD54 and CD106 via activation of the transcription factors nuclear factor-kappaB (NF-\(\kappa\)B) and activator protein 1 (AP-1) in human endothelium.\textsuperscript{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.\textsuperscript{51} 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-administered 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/\(\mu\)g protein, whereas in PBMCs of LPS-administered mice was under the quantification limit (U.L.Q. < 0.561 fmol/\(\mu\)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,\textsuperscript{20} anti-CD54,\textsuperscript{15,35,36} as well as by single treatment with anti-LFA-1 (CD11a/CD18 complex)\textsuperscript{71} antibodies, whereas anti-CD106\textsuperscript{40} and anti-VLA-4 (CD29/CD49d complex)\textsuperscript{71} antibodies had modest effect. Furthermore, anti-CD54 antibody reduced neutrophil accumulation in lung of cecal ligation puncture (CLP)-septic systemic inflammation mice, but the paracellular route across the BBB in LPS-administered mice by affecting the decreased levels of CD9, CD49c and CD97 are involved in reduced expression of CD9, CD49c and CD97 are involved in the decreased expression of CD9, CD49c and CD97 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.\textsuperscript{51} Leukocyte migration across the BBB is considered to occur via not only a transcellular route, but also a paracellular route.\textsuperscript{34,55} 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.

 Activation of integrins such as CD11a and CD18 on leukocytes is a crucial step of leukocyte migration across the BBB.\textsuperscript{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.\textsuperscript{48} On the other hand, it has been reported that the activation of CAMs such as CD54 and CD106 involves increased expression levels.\textsuperscript{9,19,20,41} From the viewpoint of the absolute protein levels obtained in the present study, the expression of CD54 (1.06 fmol/\(\mu\)g protein) in Bcap is less than that of CD11a/CD18 (3.58 and 3.15 fmol/\(\mu\)g protein, respectively) in PBMCs under normal conditions, whereas the expression of CD54 (6.56 fmol/\(\mu\)g protein) exceeds that of CD11a/CD18 (2.04 and 2.59 fmol/\(\mu\)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,\textsuperscript{42} CD26,\textsuperscript{43} CD31,\textsuperscript{44} CD49a,\textsuperscript{45} CD49c,\textsuperscript{45} CD49f,\textsuperscript{45} CD71,\textsuperscript{46} CD82,\textsuperscript{47} CD97,\textsuperscript{47} and CD98\textsuperscript{48} at the BBB are consistent with previous findings. Although protein expression of CD90, which is predominantly expressed in brain pericytes,\textsuperscript{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).\textsuperscript{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.\textsuperscript{51} We have recently reported that claudin-5 and claudin-11 are expressed at the human and rodent BBB.\textsuperscript{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.\textsuperscript{51} Leukocyte migration across the BBB is considered to occur via not only a transcellular route, but also a paracellular route.\textsuperscript{34,55} 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/\(\mu\)g protein) and CD98 (4F2hc, 13.0 fmol/\(\mu\)g protein) in Bcap of control ddY mouse determined by using CD-1 and CD-II standard proteins were consistent with reported expression levels of CD71 (4.34–5.84 fmol/\(\mu\)g protein)\textsuperscript{22,56} and CD98 (16.4–17.2 fmol/\(\mu\)g protein)\textsuperscript{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, 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 cord and (ii) CD54 is expressed in localized regions of EAE mouse brain. 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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