

# CLMP Promotes Leukocyte Migration Across Brain Barriers in Multiple Sclerosis

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## Abstract

### Background and Objectives

In multiple sclerosis (MS), peripheral immune cells use various cell trafficking molecules to infiltrate the CNS where they cause damage. The objective of this study was to investigate the involvement of coxsackie and adenovirus receptor–like membrane protein (CLMP) in the migration of immune cells into the CNS of patients with MS.

### Methods

Expression of CLMP was measured in primary cultures of human brain endothelial cells (HBECs) and human meningeal endothelial cells (HMECs), postmortem brain samples, and peripheral blood mononuclear cells (PBMCs) from patients with MS and controls by RNA sequencing, quantitative PCR, immunohistochemistry, and flow cytometry. In vitro migration assays using HBECs and HMECs were performed to evaluate the function of CLMP.

### Results

Using bulk RNA sequencing of primary cultures of human brain and meningeal endothelial cells (ECs), we have identified CLMP as a new potential cell trafficking molecule upregulated in inflammatory conditions. We first confirmed the upregulation of CLMP at the protein level on TNF $\alpha$ -activated and IFN $\gamma$ -activated primary cultures of human brain and meningeal ECs. In autopsy brain specimens from patients with MS, we demonstrated an overexpression of endothelial CLMP in active MS lesions when compared with normal control brain tissue. Flow cytometry of human PBMCs demonstrated an increased frequency of CLMP<sup>+</sup> B lymphocytes and monocytes in patients with MS, when compared with that in healthy controls. The use of a blocking antibody against CLMP reduced the migration of immune cells across the human brain and meningeal ECs in vitro. Finally, we found CLMP<sup>+</sup> immune cell infiltrates in the perivascular area of parenchymal lesions and in the meninges of patients with MS.

### Discussion

Collectively, our data demonstrate that CLMP is an adhesion molecule used by immune cells to access the CNS during neuroinflammatory disorders such as MS. CLMP could represent a target for a new treatment of neuroinflammatory conditions.

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## Glossary

**ALCAM** = activated leukocyte cell adhesion molecule; **BBB** = blood-brain barrier; **BMB** = blood-CSF meningeal barrier; **CAM** = cellular adhesion molecule; **CAR** = coxsackie and adenovirus receptor; **CLMP** = CAR-like membrane protein; **EC** = endothelial cell; **HBEC** = human brain EC; **HMEC** = human meningeal EC; **ICAM-1** = intercellular adhesion molecule 1; **IFN $\gamma$**  = interferon gamma; **IgSF** = immunoglobulin superfamily; **JAM** = junctional adhesion molecule; **MS** = multiple sclerosis; **ORO** = oil red O; **PBMC** = peripheral blood mononuclear cell; **TBST** = tris-buffered saline–0.1% Tween; **TNF $\alpha$**  = tumor necrosis factor alpha; **VCAM-1** = vascular cell adhesion molecule 1.

Multiple sclerosis (MS), the most common chronic neuro-inflammatory disorder, is characterized by activated leukocyte trafficking into the CNS, leading to demyelination. Infiltration of immune cells into the CNS is a highly regulated process that occurs both through the blood-brain barrier (BBB) and the blood-CSF meningeal barrier (BMB). During this process, adhesion of the leukocytes is mediated by interactions between cellular adhesion molecules (CAMs) expressed on the surface of the barrier endothelial cells (ECs) and their counterligand on the migrating leukocytes. These include intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), activated leukocyte cell adhesion molecule (ALCAM), dual immunoglobulin domain-containing cell adhesion molecule, and melanoma cell adhesion molecule.<sup>1-7</sup> Characterizing cell trafficking into the CNS in MS has already led to the development of an anti-VLA4 antibody, natalizumab, as a highly effective therapy for relapsing MS. Given the different profiles of immune cells and CNS barriers involved in MS, additional investigation to expand our current understanding of the cell trafficking mechanisms underlying MS pathogenesis is necessary.

Several junctional adhesion molecules (JAMs) belonging to the immunoglobulin superfamily (IgSF) were shown to be involved in transendothelial leukocyte migration. JAM-A, JAM-B, and JAM-C and JAM-related proteins such as JAM-L and the coxsackie and adenovirus receptor (CAR) were all described to be involved in the adhesion or migration of at least 1 leukocyte population or subpopulation across the brain endothelium.<sup>8-15</sup> CAR-like membrane protein (CLMP) is another member of the IgSF that is structurally related to the JAMs by sharing a similar domain organization: 2 extracellular Ig domains, a transmembrane segment, and a cytoplasmic tail.<sup>16,17</sup> Unlike the aforementioned JAMs, CLMP function in neuroinflammation has not been defined. Based on structural similarity with CAR and on experiments with CLMP-transfected cells *in vitro*, it is generally accepted that CLMP shows homotypic and heterotypic interactions, however, with unknown ligands.<sup>18</sup>

In this study, we performed bulk RNA sequencing on inflamed primary cultures of human BBB and BMB ECs and identified CLMP as a potential cell trafficking molecule involved in MS. The upregulation of CLMP on brain ECs in neuroinflammation was confirmed both at the protein level and in MS lesions. In parallel, we detected increased frequencies of several CLMP<sup>+</sup> immune cell subsets in the peripheral blood in patients with MS.

## Methods

### Human BBB and Meningeal ECs Isolation and Culture

BBB and meningeal ECs were isolated from patients undergoing epilepsy surgery. Informed consent was obtained before surgeries (CHUM research ethic committee approval number BH07.001, 20.332). Meninges were removed, and brain material was washed with PBS to remove blood. Brain material was homogenized, and cells were isolated using 350 and 112  $\mu\text{m}$  pore size mesh. For meningeal EC culture, meninges were removed from the aforementioned brain materials and then digested mechanically and chemically using collagenase type IV (2 mg/mL; Sigma-Aldrich) for 15 minutes at 37°C. Following this, ECs were isolated using a 30- $\mu\text{m}$  filter (Miltenyi). BBB and meningeal ECs were cultured at 37°C in 6-well plates coated with 0.5% gelatin in culture media composed of M199 cell culture media (ThermoFisher Scientific), 10% fetal bovine serum, 5% human normal serum (Gemini), 0.2% insulin-transferrin-sodium selenite  $\times 100$  (Sigma-Aldrich), and 0.14% EC growth supplement (BD Biosciences). The purity and characterization of these BBB and meningeal ECs was performed as previously published.<sup>19,20</sup>

### Flow Cytometry

Peripheral blood mononuclear cells (PBMCs) were collected from blood samples as previously described<sup>5</sup> after obtaining informed consent in accordance with institutional guidelines (CHUM research ethic committee approval number BH07-001, 20.332). Extracellular staining was conducted as previously described.<sup>5</sup> In brief, Fc receptors were blocked with mouse IgG isotype control (Thermo Fisher Scientific) for 20 minutes at 4°C. Cells were then incubated with LIVE/DEAD fixable Aqua dead cell stain kit (Thermo Fisher Scientific) for 15 minutes. Cells were incubated 30 minutes at 4°C for labeling with surface antigen antibodies. Doublets and dead cells were excluded using classical gating strategy on FlowJo software (FlowJo, LLC) and the stain with the viability marker. Positive gates were defined using isotype controls. The following antibodies against human cell surface markers were used: CD3, CD4, CD8, CLMP, CD14, CD19, CD45RA, and CD45RO (all from BD Biosciences).

### Immunostaining

#### Immunohistostaining

Human brain tissue was obtained from patients diagnosed with clinical and neuropathologic MS diagnosis according to

the revised 2010 McDonald criteria. Tissue samples were collected from 3 control donors (42-year-old man and 67-year-old man; 34-year-old woman) and 7 patients with MS (33-year-old and 51-year-old women with relapsing-remitting MS; 26-year-old man with relapsing-remitting MS; 48-year-old and 65-year-old men with secondary progressive MS; and 55-year-old and 60-year-old women with secondary progressive MS) with full ethical approval (BH07.001) and informed consent as approved by the local ethics committee. Autopsy samples were preserved and lesions classified using luxol fast blue/hematoxylin & eosin (LHE) staining and oil red O (ORO) staining as previously published.<sup>21-23</sup>

Frozen brain tissues were sectioned into slices (7  $\mu$ m) and fixed in acetone for 10 minutes, transferred to ethanol for 5 minutes, hydrated in PBS, and blocked with 10% species-specific serum of the secondary antibody hosts. Sections were incubated for 1 hour at room temperature with primary antibodies diluted in 3% species-specific serum. Primary antibodies were revealed by using secondary antibodies linked to AF488 or Cy3 (Thermo Fisher Scientific, dilution 1/600, incubation time 45 minutes at room temperature). Sections were then mounted using Mowiol reagent containing TO-PRO<sup>®</sup>3 (Thermo Fisher Scientific, 1/400). The following primary antibodies were used: sheep antihuman/mice CLMP (1/100; R&D Systems), rabbit antihuman laminin (1/500, Dako), mouse antihuman CD4 (1/100; BD bioscience), mouse antihuman CD8 (1/100; BD bioscience), rabbit antihuman CD20 (1/200, Abcam), and mouse antihuman CD68 (1/400; Dako).

### Immunocyto staining

Human BBB ECs were grown to confluency in 24-well plastic chamber for 4 days with 40% astrocyte-conditioned media and stimulated with a mix of TNF $\alpha$  and IFN $\gamma$  (100 U/mL) the last day. Cells were washed with PBS, fixed with 70% ethanol, and then labeled with antibodies for CLMP (1/100; R&D Systems) and mouse antihuman p120 catenin (1/50; BD bioscience).

PBMCs were cytopun in 25% glycerin and fixed in acetone for 20 seconds at room temperature. The cells were then labeled with antibodies for CD4, CD8, CD19, CD14 (mouse monoclonal; BD biosciences; 1/100), and CLMP (sheep; R&D Systems; 1/100) for 1 hour at room temperature. Primary antibodies were revealed by using secondary antibodies linked to AF488 or Cy3 (Thermo Fisher Scientific; 1/600; incubation time 45 minutes at room temperature). Sections were then mounted using Mowiol reagent containing TO-PRO<sup>®</sup>3 (Thermo Fisher Scientific, 1/400).

### Image Acquisition

Images (z-stacks) were acquired using a Leica SP5 confocal microscope with Leica LAS AF software and processed using Fiji and LAS X. All settings were kept the same.

## RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction

Confluent human ECs were stimulated for 24 hours with IL-1 $\beta$  (1 ng/mL, R&D system), IL-17A (100 ng/mL, R&D system), or a mix of TNF- $\alpha$  and IFN $\gamma$  (100 U/mL Thermo Fisher). Untreated confluent human BBB or BMB ECs were used as control.

RNA was extracted with TRIzol<sup>®</sup> reagent (Thermo Fisher Scientific) and the RNeasy Mini kit (Qiagen). RNA was transcribed into cDNA using a reverse transcription kit (Qiagen). The primers CLMP (Hs00956747\_m1; TaqMan<sup>®</sup> FAM<sup>™</sup> labeled; Life Technologies) and 18S ribosomal RNA (VIC<sup>®</sup>-labeled probe; Life Technologies) were used to assess relative gene expression levels according to the manufacturer's instructions. The CT method was used to compare levels of mRNA (cycle threshold: cycle at which gene of interest is detected in a linear range; CT = difference between the cycle threshold of the gene of interest and the cycle threshold of the internal gene control).

### Western Blot

Confluent human BBB ECs were stimulated for 24 hours with a mix of TNF $\alpha$  and IFN $\gamma$  (100 U/mL Thermo Fisher). Untreated confluent human BBB ECs were used as control. Cells were lysed with RIPA buffer with  $\times$ 1 proteinase inhibitor (Thermo Fisher) for protein isolation and kept on ice for 30 minutes. After centrifugation, the protein quantification of the supernatant was performed with the BCA protein assay kit (Thermo Fisher Pierce). SDS-PAGE (Bio-Rad) was performed by loading 10  $\mu$ g of denatured protein per lane and blotted onto a polyvinylidene difluoride membrane (Bio-Rad). After blocking with 5% milk in tris-buffered saline-0.1% Tween (TBST), the primary antibody was applied (anti-CLMP; R&D system; 1/200), followed by washes with TBST and by incubation with anti-sheep IgG horseradish peroxidase-conjugated secondary antibody (R&D system, 1/10,000). Protein bands were developed with the ECL substrate (Amersham, ECL Plus detection kit) and were then measured by densitometry using Bio-Rad Gel Doc System and Quantity One<sup>®</sup> software.

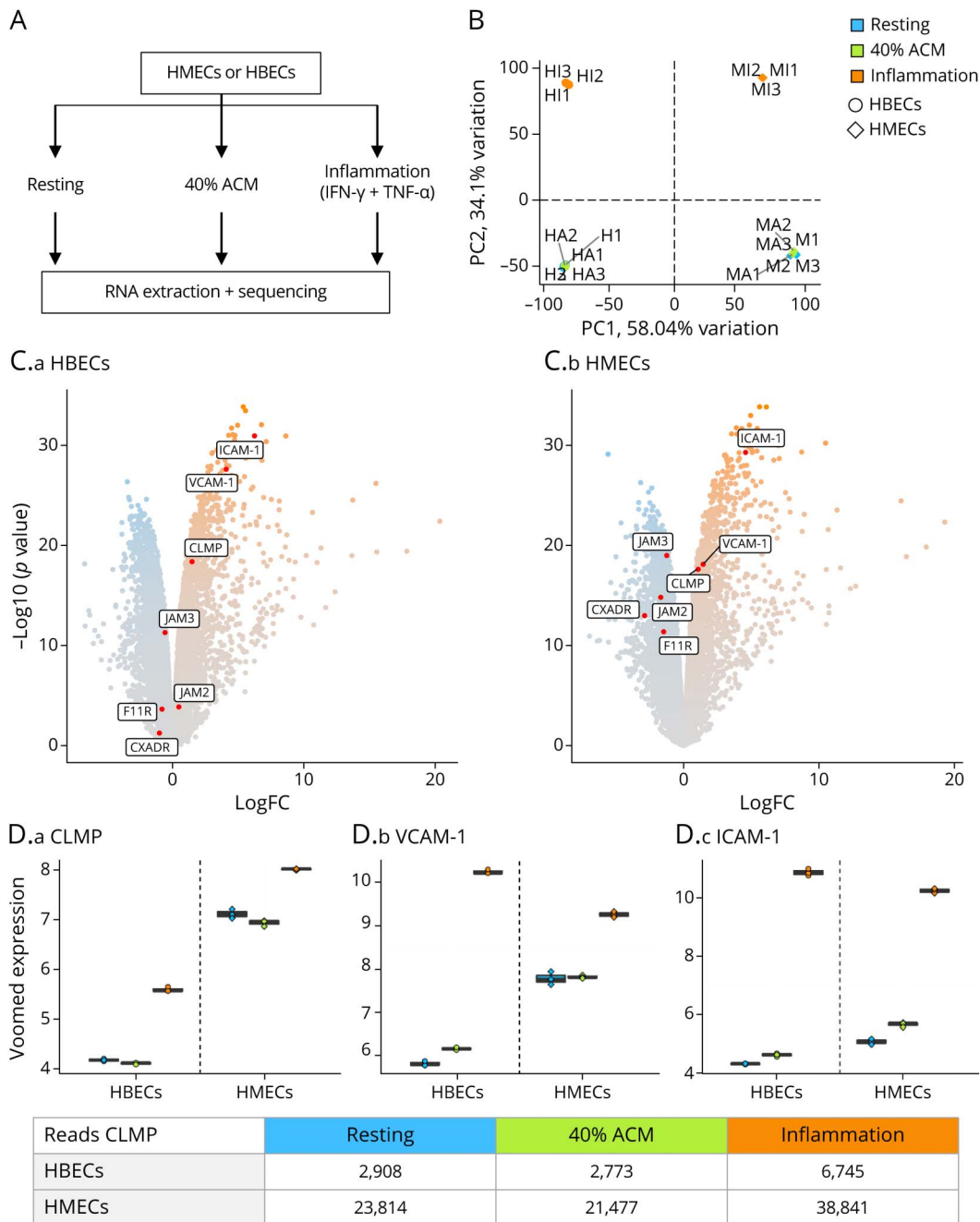
### Migration Assays

Human BBB or BMB ECs were grown for 3 days in Boyden chambers (Falcon/BD Biosciences; pore size: 3  $\mu$ m) in culture media supplemented with 40% astrocyte culture media, and cells were stimulated with a mix of TNF $\alpha$  and IFN $\gamma$  (100 U/mL) for 24 hours. Antibodies for CLMP (40  $\mu$ g/mL; sheep polyclonal, R&D Systems) or isotype control (40  $\mu$ g/mL; sheep polyclonal, R&D Systems) were added 1 hour before the start of the migration assay. Subsequently, 1 million isolated PBMCs were added to the upper chamber. After 18 hours, migrated cells in the lower chamber were counted and were then stained with antibodies against CD3, CD4, CD8, CD45RO, CD14, and CD19 for flow cytometry analyze.

### Adhesion Assay

Ninety-six-well plates were coated with 10  $\mu$ g/mL BSA, CLMP, or ICAM-1 (all from R&D systems) overnight at 4°C.

**Figure 1** Bulk RNA Sequencing Identifies CLMP as an Inflammatory Candidate Involved in Human Transendothelial Cell Trafficking



(A) RNA sequencing study design. HMECs and HBECs were stimulated for 24 hours with 40% ACM or with TNF $\alpha$  and IFN $\gamma$ . RNA was isolated at the same time point for all the conditions and samples were sequenced. (B) PCA of the 14,637 most expressed genes across all 18 samples. (C) Volcano plots showing differential expression testing for all genes between untreated and TNF- $\alpha$  and IFN- $\gamma$  treated (C.a) HBECs and (C.b) HMECs. (D) Dot plot of the RNA expression of (D.a) CLMP, (D.b) VCAM-1, and (D.c) ICAM-1 in the different conditions of stimulation (nonstimulated, astrocyte culture medium or TNF- $\alpha$ /IFN- $\gamma$ ). H = HBECs; M = HMECs. Table: Number of reads for CLMP in the different conditions. ACM = astrocyte-conditioned medium; CLMP = CAR-like membrane protein; ECs = endothelial cells; HBECs = human brain ECs; HMECs = human meningeal ECs; ICAM-1 = intercellular adhesion molecule 1; IFN $\gamma$  = interferon gamma; PCA = principal component analysis; TNF $\alpha$  = tumor necrosis factor alpha; VCAM-1 = vascular cell adhesion molecule 1.

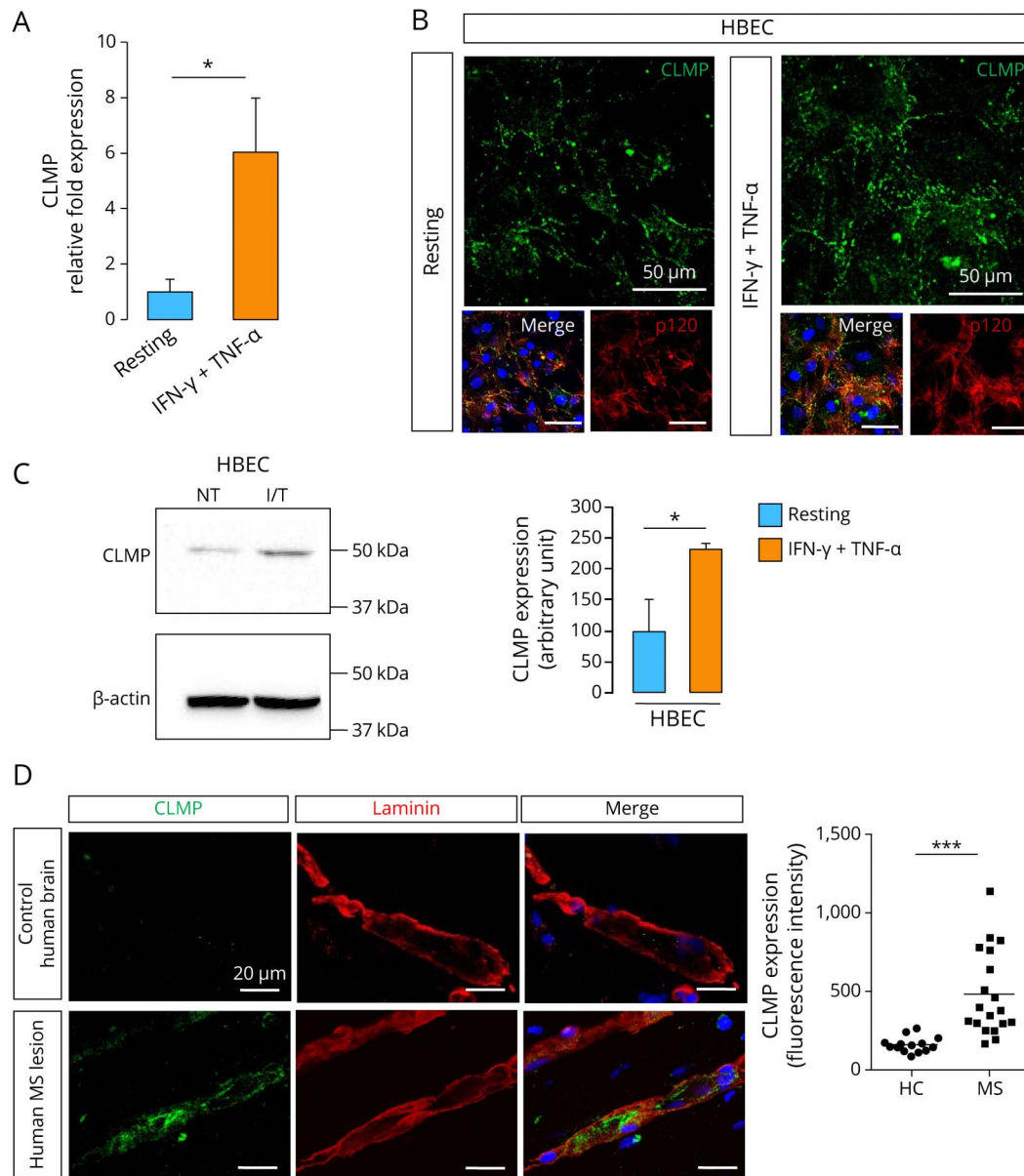
In vitro-activated human T lymphocytes, labeled with 5, 6-carboxyfluorescein diacetate succinimidyl ester (Molecular Probes, Thermo Fisher Scientific), were then transferred to the coated wells in triplicates at 100,000 cells per well. After a 4-hour incubation period at 37°C, non-adherent cells were removed, wells were gently washed

twice with PBS, and fluorescence was measured (Synergy 4, Biotek).

### Bulk RNA Seq

Bulk RNA Seq of the 18 EC samples was conducted on Illumina HiSeq platform. Quality of isolated RNA was

**Figure 2** CLMP is Upregulated by Human Brain Endothelial Cells Under Inflammatory Condition and in MS Lesions



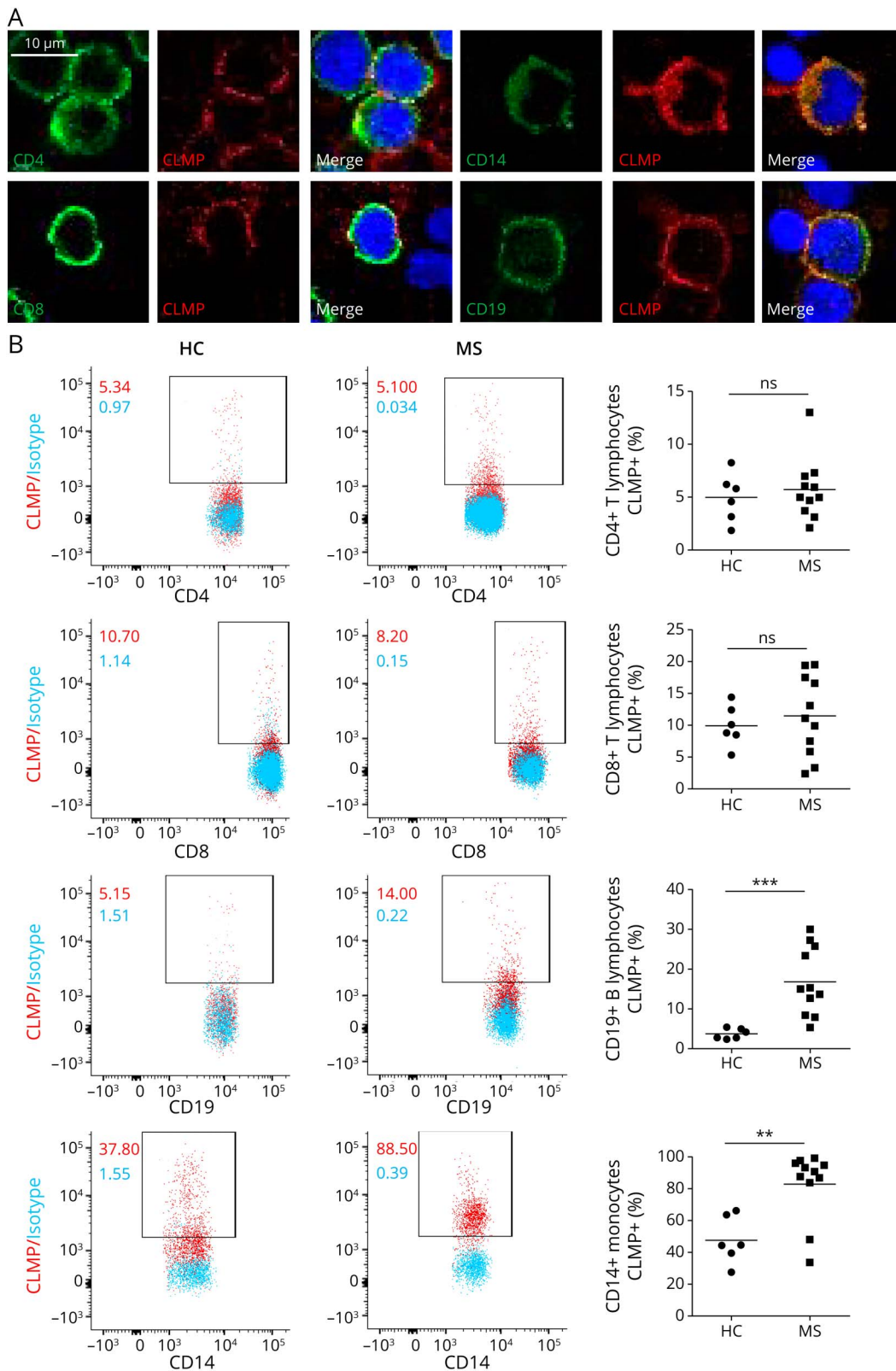
(A) Quantitative PCR analysis of CLMP mRNA expression relative to 18S ribosomal RNA in HBECs and HMECs ( $n = 8$ ; mean SEM;  $*p < 0.05$ , by the Student  $t$  test). (B) CLMP (green) and p120 (red) on the surface of HBECs left untreated or treated for 24 hours with TNF- $\alpha$  and IFN- $\gamma$ ; nuclei are stained with TOPRO-3 (blue). Scale bar: 50  $\mu\text{m}$ . Representative of  $n = 4$  independent experiments. (C) Expression of CLMP protein assessed by Western blot (WB) in HBECs left untreated or treated for 24 hours with TNF- $\alpha$  and IFN- $\gamma$ . Representative of  $n = 3$  different preparations. Corresponding quantification of WB signal intensity ( $n = 3$ –4; mean SEM;  $*p < 0.05$ , by the Student  $t$  test). (D) Immunohistochemistry with confocal microscopy of CLMP (green) in vessels (laminin, red) of control brains and MS active lesions. Scale bar = 20  $\mu\text{m}$ ; data shown are representative of  $n = 3$ –4. Corresponding quantification of CLMP fluorescence intensity in CNS vessels in healthy brains and MS active lesions ( $n = 3$ –4;  $***p < 0.001$ , by the Student  $t$  test). CLMP = CAR-like membrane protein; ECs = endothelial cells; HBECs = human brain ECs; HMECs = human meningeal ECs; IFN $\gamma$  = interferon gamma; MS = multiple sclerosis; TNF $\alpha$  = tumor necrosis factor alpha.

assessed using bioanalyzed and nanodrop. Library preparation was performed with Illumina TruSeq kit. Processing of the sequencing raw files was performed following the MUGQIC RNA-Seq pipeline. The pipeline uses STAR to align reads to the genome. Picard was used to produce gene expression matrix. R packages DESeq2 and edgeR were used to perform downstream analysis of gene expression.

### Statistical Analysis

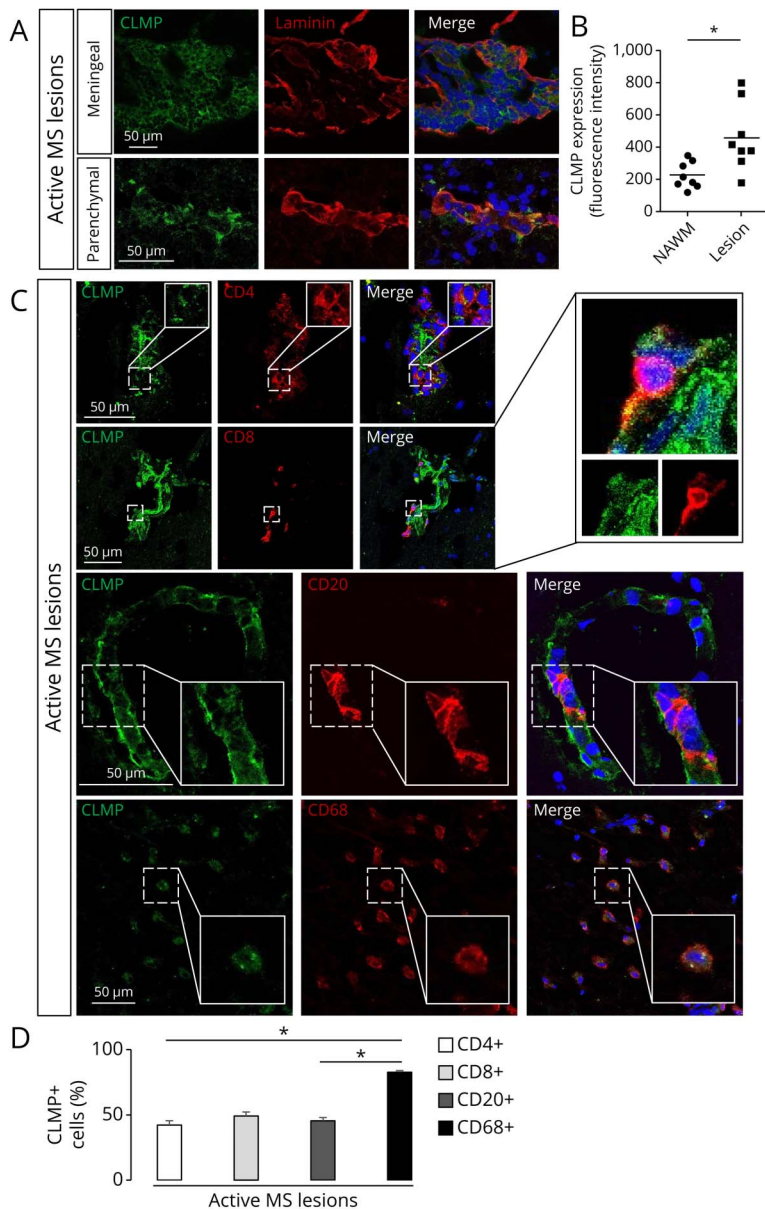
Graphpad Prism and R software (The R Foundation for Statistical Computing, [www.R-project.org](http://www.R-project.org)) were used for statistical analyses. Results are presented as mean  $\pm$  standard error of the mean. Paired or unpaired Student  $t$  tests, Wilcoxon-Mann-Whitney tests, or Wilcoxon matched-pairs signed rank tests were performed. One-way analysis of variance tests were performed, followed by the appropriate post hoc test when more than 2 groups were compared.

**Figure 3** MS Is Associated With Increased Frequency of CLMP+/CD19+ B Lymphocytes and CLMP+/CD14+ Monocytes Ex Vivo



(A) Representative cytospin images of PBMCs immunostained for CD4, CD8, CD19, CD14 (red), CLMP (green), and nuclei (TOPRO-3; blue) and acquired by confocal microscopy. Scale bar: 20  $\mu$ m. (B) Representative dot plots and quantification of flow cytometry analysis of CLMP expression by CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, CD19<sup>+</sup> B lymphocytes, and CD14<sup>+</sup> monocytes obtained from peripheral blood of healthy controls or patients with MS; corresponding quantification (n = 6–11; ns = not significant; \*\* $p$  < 0.01; \*\*\* $p$  < 0.001; the Wilcoxon-Mann-Whitney test). CLMP = CAR-like membrane protein; MS = multiple sclerosis; PBMCs = peripheral blood mononuclear cells.

**Figure 4** CLMP+ Immune Cells Invade the CNS in Patients With MS



(A) Representative immunofluorescent images of tissue sections of meningeal and parenchymal infiltrates in active MS brain lesions. Stainings are for CLMP (green), laminin, (red), and nuclei (TOPRO). Scale bar = 50  $\mu$ m. (B) Quantification of CLMP expression in patients with MS in lesion area or normal-appearing white matter (NAWM); n = 16 areas from 4 different patients with MS (\* $p < 0.05$ , by Student *t* test). (C) Representative immunofluorescent images of frozen sections of immune cell infiltrates in active MS brain lesions. Stainings are for CLMP (green), CD4, CD8, CD68, CD20 (red), and nuclei (TOPRO). Scale bar = 50  $\mu$ m. (D) Quantification in active MS lesions of CD4<sup>+</sup>, CD8<sup>+</sup>, CD20<sup>+</sup>, and CD68<sup>+</sup> cells that are positive for CLMP, by confocal microscopy; n = 11–17 area from 3 different patients with MS (\* $p < 0.05$  by 1-way ANOVA with the Tukey post hoc test). ANOVA = analysis of variance; CLMP = CAR-Like membrane protein; MS = multiple sclerosis.

Values were considered statistically significant when  $p < 0.05$ .

### Standard Protocol Approvals, Registrations, and Patient Consents

Informed consent was obtained before surgeries (CHUM research ethic committee approval number BH07.001, 20.332).

### Data Availability

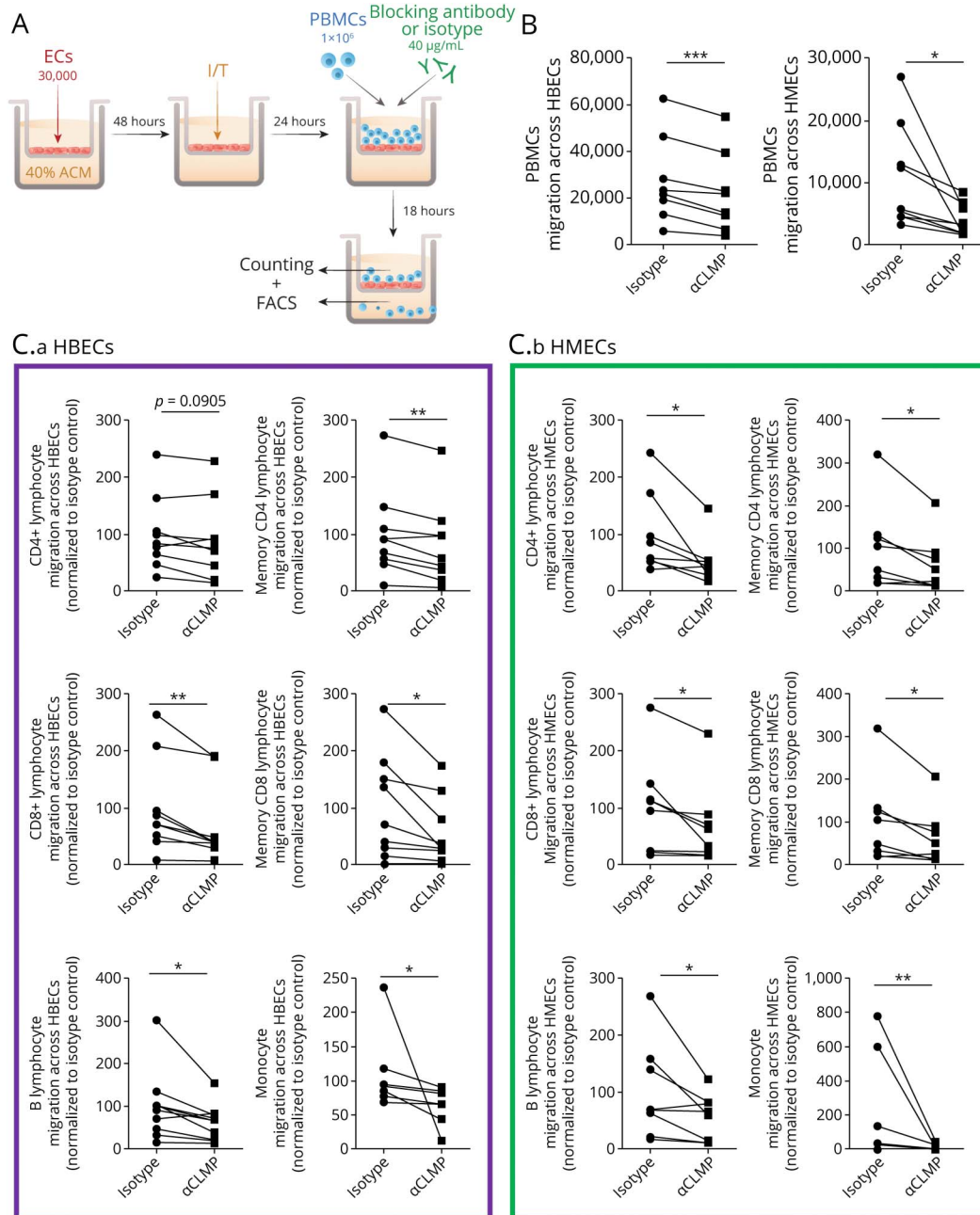
The data that support the findings of this study can be shared at the request of other investigators for the purpose of replicating procedures and results.

## Results

### CLMP Is Upregulated on Human Brain ECs Under Inflammatory Condition and in MS Lesions

CLMP is upregulated on human brain ECs (HBECs) under inflammatory condition and in MS lesions. To identify new players involved in neuroinflammatory immune cell trafficking in MS, we performed bulk RNA sequencing of resting and inflamed primary cultures of HBECs and human meningeal ECs (HMECs) (Figure 1A). As proinflammatory cytokines are known to induce an upregulation of various adhesion molecules on ECs,<sup>3,4,7,15,24</sup> we stimulated HBECs and HMECs with tumor necrosis factor alpha (TNF $\alpha$ ) and interferon gamma (IFN $\gamma$ ) that are 2 cytokines

**Figure 5** CLMP Mediates the Migration of Human Immune Cells Across Human CNS Barriers



(A) Experimental design. Human ex vivo PBMCs from healthy donors were allowed to migrate for 18 hours across primary cultures of inflamed HBECs or HMECs, using a modified Boyden chamber model. PBMCs and ECs were preincubated for 1 hour with isotype control vs blocking antibody against CLMP. (B) Migration is expressed as absolute number of cells in the lower chamber. (C) Frequencies of migrated PBMCs across HBECs and HMECs; Data shown are pooled from 4 independent experiments (n = 8–9 blood donors in total; \* $p < 0.05$ ; \*\* $p < 0.01$ ; (B) paired  $t$  test; (C) paired  $t$  test or the Wilcoxon matched-pairs signed rank test) performed in triplicate using 4 distinct (C.a) HBEC preparations and 4 distinct (C.b) HMEC preparations. ACM = astrocyte-conditioned medium; CLMP = CAR-like membrane protein; ECs = endothelial cells; HBECs = human brain ECs; HMECs = human meningeal ECs; I/T = IFN $\gamma$  and TNF $\alpha$ ; PBMCs = peripheral blood mononuclear cells; TNF $\alpha$  = tumor necrosis factor alpha.

elevated in lesions and CSF of patients with MS.<sup>25-27</sup> Astrocyte-conditioned medium was used as a positive control to induce BBB properties in ECs.<sup>28</sup> We first used a principal component analysis to explore the variance captured by quantifying gene expression in the ECs. Cell types (HMECs and HBECs) were segregated along the major component (PC1), and the second principal component (PC2) was associated with the EC response to inflammation

with IFN $\gamma$ /TNF $\alpha$  (Figure 1B). Among the upregulated genes, we identified CLMP as a potentially major CNS cell trafficking molecule because it was differentially expressed in both HBECs and HMECs (HBECs: logFC = 1.42, false discovery rate = 9.98e-18; HMECs: logFC = 0.89, false discovery rate = 2.13e-15; Figure 1, C and D) and was previously reported to perform cell-cell adhesion functions in a nonimmunologic context.<sup>29</sup>



To validate our initial findings, we performed quantitative real-time PCR on resting and inflamed HBECs and HMECs and observed a significant upregulation in *CLMP* mRNA expression on inflammation compared with that in resting condition (Figure 2A, and eFigure 1A, links.lww.com/NXI/A740). At the protein level, CLMP was found to be significantly increased in inflamed ECs both by immunofluorescence and Western blot analyses (Figure 2, B and 2C and eFigure 1B, links.lww.com/NXI/A740). To establish the relevance of our in vitro observations in MS, we evaluated CLMP expression on ECs in CNS lesions of patients with MS and found a strong upregulation of CLMP on the surface of CNS vessels in active MS lesions when compared with that in controls (Figure 2D). These results demonstrate that CLMP is upregulated on brain ECs under inflammatory conditions in vitro and in MS lesions in situ.

### CLMP Is Expressed by Human Peripheral Blood Leukocytes

In inflammatory conditions, immune cells circulating in the blood can upregulate specific surface proteins promoting interactions with BBB or BMB ECs and subsequent invasion of the CNS. Because CLMP was demonstrated to form homotypic interactions,<sup>29,30</sup> we decided to characterize its expression by ex vivo PBMCs. Using immunofluorescence microscopy, we observed that CLMP is indeed expressed on different immune cell populations such as CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, CD14<sup>+</sup> monocytes, and CD19<sup>+</sup> B lymphocytes in healthy donors (Figure 3A). To further quantify CLMP expression on immune cells and define their relevance for MS, we performed flow cytometry analyses on PBMCs obtained from healthy donors and patients with MS. The proportion of CLMP<sup>+</sup>/CD4<sup>+</sup> and CLMP<sup>+</sup>/CD8<sup>+</sup> T lymphocytes were not significantly different in healthy donors and patients with MS (4.97% ± 2.29% vs 5.71% ± 2.89% for CD4<sup>+</sup> T lymphocytes and 9.92% ± 3.18% vs 11.47% ± 6.25% for CD8<sup>+</sup> T lymphocytes; n = 6–11; Figure 3B). A deeper analysis of these cells in patients with MS showed that CLMP expression is approximately 2 times greater in ex vivo memory CD45RO<sup>+</sup> than in naïve CD45RA<sup>+</sup> T lymphocytes (eFigure 2, links.lww.com/NXI/A740). However, the proportion of CD19<sup>+</sup> B lymphocytes and CD14<sup>+</sup> monocytes expressing CLMP were strikingly increased in patients with MS (3.75% ± 1.28% vs 16.8% ± 8.49% for B lymphocytes, and 47.63% ± 14.77% vs 82.9% ± 21.53% for monocytes; n = 6–11; Figure 3B). Together, our observations demonstrate that CLMP is expressed on human leukocytes in the peripheral blood and that the frequency of CLMP<sup>+</sup> B lymphocytes and monocytes is increased in patients with MS rather than in healthy donors.

### CLMP<sup>+</sup> Leukocytes Are Found in Active Brain Lesions of Patients With MS

The increased expression of CLMP on peripheral leukocytes and brain ECs in patients with MS strongly suggests

its involvement in leukocyte trafficking across the BBB and BMB in MS. To address this hypothesis, we first assessed the presence of CLMP<sup>+</sup> leukocytes in situ in active MS lesions. Autopsy-derived MS CNS material was stained with luxol fast blue hematoxylin and eosin (eFigure 3A, links.lww.com/NXI/A740) and ORO (eFigure 3B) to identify lesion type. Globally, we found the expression of CLMP to be significantly higher in lesion areas with active infiltration than in areas without infiltration in both the meninges and in the brain parenchyma of patients with MS (Figure 4, A and B; n = 16 different areas from 4 patients with MS). A deeper analysis of CLMP expression on MS lesions revealed the presence of CLMP on CD4<sup>+</sup> T lymphocytes, CD8<sup>+</sup> T lymphocytes, CD68<sup>+</sup> macrophages and CD20<sup>+</sup> B lymphocytes near blood vessels (Figure 4C). In accordance with the flow cytometry results showing that monocytes are the immune cell-type showing the highest proportion of CLMP<sup>+</sup> cells (Figure 3B), the proportion of CD68<sup>+</sup> macrophages expressing CLMP in MS lesions is higher than that for the other immune cell-types (Figure 4D). By detecting the increased presence of different CLMP<sup>+</sup> immune cells in active MS lesions, our data suggest that CLMP could mediate immune cell trafficking into the CNS in MS.

### Blocking CLMP With a Neutralizing Antibody Impairs Human Immune Cell Diapedesis Across Brain Barrier Endothelium

To accumulate in MS brains, immune cells must first cross the endothelia of the different brain barriers.<sup>31,32</sup> Because CLMP is an adhesion molecule with conserved Ig domains that mediate cell-cell adhesion through homotypic interaction,<sup>29</sup> we investigated its role in the transmigration of leukocytes across both BBB and BMB ECs in vitro. In brief, leukocytes isolated from the peripheral blood of healthy donors were allowed to migrate in the presence of anti-CLMP or isotype antibodies across confluent monolayers of inflamed HBECs and HMECs cultured in Boyden chambers (Figure 5A). Blocking CLMP resulted in a significant decrease of diapedesis of CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, B lymphocytes, and monocytes across HBECs and HMECs (Figure 5, B and C, n = 6–9). Flow cytometric analysis of monocytes in the lower chambers (migrated cells) vs upper chambers (nonmigrated cells) demonstrated that CLMP is preferentially expressed on monocytes that migrated across ECs (eFigure 4A, links.lww.com/NXI/A740). To confirm that significant interactions can take place between leukocyte and CLMP, we performed an adhesion assay and found that adhesion of human T lymphocytes to coated recombinant human (rh)CLMP was significantly higher than to BSA 1% but comparable with adhesion to coated ICAM-1. The blocking of CLMP resulted in a suppression of cell adhesion to (rh)CLMP but not to ICAM-1 (eFigure 4B, links.lww.com/NXI/A740). These results demonstrate that CLMP is directly involved in the migration of human leukocytes across brain barriers.

## Discussion

Currently, although there is no cure for MS, the available treatments can significantly decrease disease activity and, to some extent, its progression.<sup>33</sup> The efficacy of the MS therapy natalizumab stems from its capacity to block the functioning of the cell trafficking molecule VLA4. However, its great capacity to block immune cell infiltration into the brain was shown to alter the CNS protective immune surveillance and can result in life-threatening adverse events,<sup>34</sup> thereby limiting natalizumab use in clinic to JCV-negative patients. Similarly, despite its clinical efficacy when treating MS, the monoclonal antibody efalizumab, which targets CD11a, the ligand of ICAM-1, was withdrawn from the market because of collateral damage of brain immune surveillance.<sup>35</sup> Discovering new molecular players involved in immune cell diapedesis across brain barriers is therefore a great unmet need for the development of safer MS treatments.

In this study, we demonstrated that human brain and meningeal ECs strongly upregulate CLMP *in vitro* after stimulation with proinflammatory cytokines. We also reported that peripheral blood leukocytes express CLMP, the frequencies of CLMP<sup>+</sup> B lymphocytes and monocytes are increased in patients with MS, and CLMP<sup>+</sup> immune cells are increased in MS lesions. Finally, we showed that blocking CLMP with an anti-CLMP antibody impairs the capacity of immune cells to cross the BBB and BMB *in vitro*. Our results support an important role for CLMP in the recruitment of immune cells into the CNS during neuroinflammation, thus suggesting a novel avenue to treat MS.

The efficacy of B-lymphocyte depletion in MS provides strong evidence of the involvement of B lymphocytes in disease activity.<sup>36</sup> Meningeal B-cell follicles are found in patients with MS and are associated with cortical inflammation and neuronal loss in progressive forms of the disease.<sup>37-39</sup> We have previously described the implication of ALCAM as an important player of B-lymphocyte infiltration in the CNS.<sup>40</sup> In this study, we demonstrated that CLMP is upregulated on meningeal ECs in inflammatory conditions and the frequency of CLMP<sup>+</sup> B lymphocytes is increased in patients with MS, suggesting that CLMP could also be involved in the accumulation of B lymphocytes in the meninges in progressive forms of MS. In line with this, the blockade of CLMP reduced human B-lymphocyte migration across meningeal ECs. Targeting CLMP in patients with MS could be a promising strategy to reduce MS progression by reducing B-lymphocyte infiltration and accumulation in the meninges.

Monocytes are also believed to play an important role in MS pathophysiology, especially in progression. After their infiltration in the CNS, monocytes can differentiate into dendritic cells or macrophages, which are widely implicated in MS neuroinflammatory processes.<sup>19,41,42</sup> Strikingly, our data demonstrated that most monocytes from the blood of patients with MS expressed CLMP vs less than half of them

in healthy donors. We also demonstrated that CLMP<sup>+</sup> monocytes are present in active lesions and the blockade of CLMP significantly reduce monocyte migration across meningeal and brain ECs. These observations suggest that a therapeutic approach to block CLMP could have a major effect in blocking monocyte migration and therefore in hindering their pathologic functions in MS.

We found that CLMP is expressed by 5% of CD4<sup>+</sup> T lymphocytes and 10% of CD8<sup>+</sup> T lymphocytes in the peripheral blood of healthy individuals and patients with MS. Surprisingly, however, the blockade of CLMP reduced the migration of T lymphocytes *in vitro* by 39.8% across the BMB endothelium and by 24.2% across the BBB endothelium. Owing to its structural similarities with CAR, which can carry homotypic and heterotypic interactions with different ligands,<sup>17</sup> we hypothesized that the CLMP-mediated T-lymphocyte trafficking across the brain barriers likely involve additional binding partners. Future work should therefore address the heterotypic interactions of CLMP on T lymphocytes and on brain ECs and unravel the contribution of each interaction to MS development. The static migration assay used is suitable to study the migration of the immune cells across ECs *in vitro*. However, cell trafficking across brain barriers is a multistep process involving rolling and adhesion of immune cells on ECs. To study the implication of CLMP in these different steps of cell trafficking, the use of *in vitro* models using shear flow and live cell imaging would be required.

In conclusion, our study demonstrates that CLMP is involved in leukocyte migration across both the BBB and BMB in MS. Its antibody-mediated blocking showcases the feasibility of its pharmaceutical targeting. The association of CLMP expression with active MS allows us to further postulate that CLMP represents a new biomarker of brain inflammation. Altogether, our study suggests that CLMP is a promising therapeutic target for MS.

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## Appendix *(continued)*

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Continued

## Appendix (continued)

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## References

- Steffen BJ, Butcher EC, Engelhardt B. Evidence for involvement of ICAM-1 and VCAM-1 in lymphocyte interaction with endothelium in experimental autoimmune encephalomyelitis in the central nervous system in the SJL/J mouse. *Am J Pathol.* 1994;145(1):189-201.
- Cannella B, Raine CS. The adhesion molecule and cytokine profile of multiple sclerosis lesions. *Ann Neurol.* 1995;37(4):424-435. doi: 10.1002/ana.410370404.
- Cayrol R, Wosik K, Berard JL, et al. Activated leukocyte cell adhesion molecule promotes leukocyte trafficking into the central nervous system. *Nat Immunol.* 2008; 9(2):137-145. doi: 10.1038/ni1551.
- Larochelle C, Cayrol R, Kebir H, et al. Melanoma cell adhesion molecule identifies encephalitogenic T lymphocytes and promotes their recruitment to the central nervous system. *Brain.* 2012;135(pt 10):2906-2924. doi: 10.1093/brain/aww212.
- Larochelle C, Lécuyer MA, Alvarez JI, et al. Melanoma cell adhesion molecule-positive CD8 T lymphocytes mediate central nervous system inflammation: MCAM+ CD8 in Neuroinflammation. *Ann Neurol.* 2015;78(1):39-53. doi: 10.1002/ana.24415.
- Schneider-Hohendorf T, Rossaint J, Mohan H, et al. VLA-4 blockade promotes differential routes into human CNS involving PSGL-1 rolling of T cells and MCAM-adhesion of TH17 cells. *J Exp Med.* 2014;211(9):1833-1846. doi: 10.1084/jem.20140540.
- Charabati M, Grasmuck C, Ghannam S, et al. DICAM promotes TH17 lymphocyte trafficking across the blood-brain barrier during autoimmune neuroinflammation. *Sci Transl Med.* 2022;14(626):eabj0473. doi: 10.1126/scitranslmed.abj0473.
- Martin-Padura I, Lostaglio S, Schneemann M, et al. Junctional adhesion molecule, a novel member of the immunoglobulin superfamily that distributes at intercellular junctions and modulates monocyte transmigration. *J Cell Biol.* 1998;142(1):117-127. doi: 10.1083/jcb.142.1.117.
- Cunningham SA, Rodriguez JM, Arrate MP, Tran TM, Brock TA. JAM2 interacts with alpha4beta1. Facilitation by JAM3. *J Biol Chem.* 2002;277(31):27589-27592. doi: 10.1074/jbc.C200331200.
- Ostermann G, Weber KSC, Zerneck A, Schröder A, Weber C. JAM-1 is a ligand of the beta(2) integrin LFA-1 involved in transendothelial migration of leukocytes. *Nat Immunol.* 2002;3(2):151-158. doi: 10.1038/ni755.
- Santoso S, Sachs UJH, Kroll H, et al. The junctional adhesion molecule 3 (JAM-3) on human platelets is a counterreceptor for the leukocyte integrin Mac-1. *J Exp Med.* 2002;196(5):679-691. doi: 10.1084/jem.20020267.
- Lamagna C, Meda P, Mandicourt G, et al. Dual interaction of JAM-C with JAM-B and alpha(M)beta2 integrin: function in junctional complexes and leukocyte adhesion. *Mol Biol Cell.* 2005;16(10):4992-5003. doi: 10.1091/mbc.e05-04-0310.
- Zen K, Liu Y, McCall IC, et al. Neutrophil migration across tight junctions is mediated by adhesive interactions between epithelial coxsackie and adenovirus receptor and a junctional adhesion molecule-like protein on neutrophils. *Mol Biol Cell.* 2005;16(6): 2694-2703. doi: 10.1091/mbc.e05-01-0036.
- Luissint AC, Lutz PG, Calderwood DA, Couraud PO, Bourdoulous S. JAM-L-mediated leukocyte adhesion to endothelial cells is regulated in cis by

- alpha4beta1 integrin activation. *J Cell Biol.* 2008;183(6):1159-1173. doi: 10.1083/jcb.200805061.
- Alvarez JI, Kébir H, Cheslow L, et al. JAM1 mediates monocyte and CD8 T cell migration across the brain endothelium. *Ann Clin Transl Neurol.* 2015;2(11):1032-1037. doi: 10.1002/acn3.255.
- Luissint AC, Nusrat A, Parkos CA. JAM-related proteins in mucosal homeostasis and inflammation. *Semin Immunopathol.* 2014;36(2):211-226. doi: 10.1007/s00281-014-0421-0.
- Matthäus C, Langhorst H, Schütz L, Jüttner R, Rathjen FG. Cell-cell communication mediated by the CAR subgroup of immunoglobulin cell adhesion molecules in health and disease. *Mol Cell Neurosci.* 2017;81:32-40. doi: 10.1016/j.mcn.2016.11.009.
- Rathjen FG. The CAR group of Ig cell adhesion proteins-regulators of gap junctions? *Bioessays.* 2020;42(12):e2000031. doi: 10.1002/bies.202000031.
- Ifergan I, Kébir H, Bernard M, et al. The blood-brain barrier induces differentiation of migrating monocytes into Th17-polarizing dendritic cells. *Brain.* 2008;131(pt 3): 785-799. doi: 10.1093/brain/awm295.
- Alvarez JI, Dodelet-Devillers A, Kebir H, et al. The Hedgehog pathway promotes blood-brain barrier integrity and CNS immune quiescence. *Science.* 2011;334(6063): 1727-1731. doi: 10.1126/science.1206936.
- Polman CH, Reingold SC, Banwell B, et al. Diagnostic criteria for multiple sclerosis: 2010 revisions to the McDonald criteria. *Ann Neurol.* 2011;69(2):292-302. doi: 10.1002/ana.22366.
- Kuhlmann T, Ludwin S, Prat A, Antel J, Brück W, Lassmann H. An updated histological classification system for multiple sclerosis lesions. *Acta Neuropathol.* 2017; 133(1):13-24. doi: 10.1007/s00401-016-1653-y.
- Dhaeze T, Tremblay L, Lachance C, et al. CD70 defines a subset of proinflammatory and CNS-pathogenic TH1/TH17 lymphocytes and is overexpressed in multiple sclerosis. *Cell Mol Immunol.* 2019;16(7):652-665. doi: 10.1038/s41423-018-0198-5.
- Luster AD, Alon R, von Andrian UH. Immune cell migration in inflammation: present and future therapeutic targets. *Nat Immunol.* 2005;6(12):1182-1190. doi: 10.1038/ni1275.
- Kebir H, Ifergan I, Alvarez JI, et al. Preferential recruitment of interferon-gamma-expressing TH17 cells in multiple sclerosis. *Ann Neurol.* 2009;66(3):390-402. doi: 10.1002/ana.21748.
- Rossi S, Motta C, Studer V, et al. Tumor necrosis factor is elevated in progressive multiple sclerosis and causes excitotoxic neurodegeneration. *Mult Scler.* 2014;20(3): 304-312. doi: 10.1177/1352458513498128.
- Selmaj K, Raine CS, Cannella B, Brosnan CF. Identification of lymphotoxin and tumor necrosis factor in multiple sclerosis lesions. *J Clin Invest.* 1991;87(3):949-954. doi: 10.1172/JCI115102.
- Yamagata K, Tagami M, Nara Y, et al. Astrocyte-conditioned medium induces blood-brain barrier properties in endothelial cells. *Clin Exp Pharmacol Physiol.* 1997;24(9-10):710-713. doi: 10.1111/j.1440-1681.1997.tb02117.x.
- Eguchi J, Wada J, Hida K, et al. Identification of adipocyte adhesion molecule (ACAM), a novel CTX gene family, implicated in adipocyte maturation and development of obesity. *Biochem J.* 2005;387(pt 2):343-353. doi: 10.1042/BJ20041709.
- Raschperger E, Engstrom U, Petterson RF, Fuxe J. CLMP, a novel member of the CTX family and a new component of epithelial tight junctions. *J Biol Chem.* 2004; 279(1):796-804. doi: 10.1074/jbc.M308249200.
- Larochelle C, Alvarez JI, Prat A. How do immune cells overcome the blood-brain barrier in multiple sclerosis? *FEBS Lett.* 2011;585(23):3770-3780. doi: 10.1016/j.febslet.2011.04.066.
- Engelhardt B, Vajkoczy P, Weller RO. The movers and shapers in immune privilege of the CNS. *Nat Immunol.* 2017;18(2):123-131. doi: 10.1038/ni.3666.
- McGinley MP, Goldschmidt CH, Rae-Grant AD. Diagnosis and treatment of multiple sclerosis: a review. *JAMA.* 2021;325(8):765-779. doi: 10.1001/jama.2020.26858.
- Stive O, Marra CM, Jerome KR, et al. Immune surveillance in multiple sclerosis patients treated with natalizumab. *Ann Neurol.* 2006;59(5):743-747. doi: 10.1002/ana.20858.
- Carson KR, Focosi D, Major EO, et al. Monoclonal antibody-associated progressive multifocal leukoencephalopathy in patients treated with rituximab, natalizumab, and efalizumab: A Review from the Research on Adverse Drug Events and Reports (RADAR) Project. *Lancet Oncol.* 2009;10(8):816-824. doi: 10.1016/S1470-2045(09)70161-5.
- Hauser SL, Waubant E, Arnold DL, et al. HERMES Trial Group. B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med.* 2008;358(7): 676-688. doi: 10.1056/NEJMoa0706383.
- Magliozzi R, Howell O, Vora A, et al. Meningeal B-cell follicles in secondary progressive multiple sclerosis associate with early onset of disease and severe cortical pathology. *Brain a J Neurol.* 2007;130(pt 4):1089-1104. doi: 10.1093/brain/awm038.
- Magliozzi R, Howell OW, Reeves C, et al. A Gradient of neuronal loss and meningeal inflammation in multiple sclerosis. *Ann Neurol.* 2010;68(4):477-493. doi: 10.1002/ana.22230.
- Howell OW, Reeves CA, Nicholas R, et al. Meningeal inflammation is widespread and linked to cortical pathology in multiple sclerosis. *Brain a J Neurol.* 2011;134(pt 9): 2755-2771. doi: 10.1093/brain/awr182.
- Michel L, Grasmuck C, Charabati M, et al. Activated leukocyte cell adhesion molecule regulates B lymphocyte migration across central nervous system barriers. *Sci Transl Med.* 2019;11(518):eaaw0475. doi: 10.1126/scitranslmed.aaw0475.
- Luessi F, Zipp F, Witsch E. Dendritic cells as therapeutic targets in neuroinflammation. *Cell Mol Life Sci.* 2016;73(13):2425-2450. doi: 10.1007/s00018-016-2170-9.
- Moreno MA, Burns T, Yao P, Miers L, Pleasure D, Soulika AM. Therapeutic depletion of monocyte-derived cells protects from long-term axonal loss in experimental autoimmune encephalomyelitis. *J Neuroimmunol.* 2016;290:36-46. doi: 10.1016/j.jneuroim.2015.11.004.