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β₂ Integrin–Mediated Crawling on Endothelial ICAM-1 and ICAM-2 Is a Prerequisite for Transcellular Neutrophil Diapedesis across the Inflamed Blood–Brain Barrier

Roser Gorina,* Ruth Lyck,* Dietmar Vestweber,† and Britta Engelhardt*

In acute neuroinflammatory states such as meningitis, neutrophils cross the blood–brain barrier (BBB) and contribute to pathological alterations of cerebral function. The mechanisms that govern neutrophil migration across the BBB are ill defined. Using live-cell imaging, we show that LPS-stimulated BBB endothelium supports neutrophil arrest, crawling, and diapedesis under physiological flow in vitro. Investigating the interactions of neutrophils from wild-type, CD11a−/−, CD11b−/−, and CD18null mice with wild-type, junctional adhesion molecule-A−/−, ICAM-1null, ICAM-2−/−, or ICAM-1null/ICAM-2−/− primary mouse brain microvascular endothelial cells, we demonstrate that neutrophil arrest, polarization, and crawling required G-protein–coupled receptor–dependent activation of β₂ integrins and binding to endothelial ICAM-1. LFA-1 was the prevailing ligand for endothelial ICAM-1 in mediating neutrophil shear resistant arrest, whereas Mac-1 was dominant over LFA-1 in mediating neutrophil polarization on the BBB in vitro. Neutrophil crawling was mediated by endothelial ICAM-1 and ICAM-2 and neutrophil LFA-1 and Mac-1. In the absence of crawling, few neutrophils maintained adhesive interactions with the BBB endothelium by remaining either stationary on endothelial junctions or displaying transient adhesive interactions characterized by a fast displacement on the endothelium along the direction of flow. Diapedesis of stationary neutrophils was unchanged by the lack of endothelial ICAM-1 and ICAM-2 and occurred exclusively via the paracellular pathway. Crawling neutrophils, although preferentially crossing the BBB through the endothelial junctions, could additionally breach the BBB via the transcellular route. Thus, β₂ integrin–mediated neutrophil crawling on endothelial ICAM-1 and ICAM-2 is a prerequisite for transcellular neutrophil diapedesis across the inflamed BBB. The Journal of Immunology, 2014, 192: 324–337.

Maintenance of homeostasis is required for proper function of neurons in the CNS. Under physiological conditions, the CNS is tightly sealed from the changeable milieu in the blood by the blood–brain barrier (BBB) and the blood–cerebrospinal fluid barrier (BCSFB). Whereas the BBB is localized at the level of highly specialized endothelial cells within CNS microvessels, the BCSFB is established by choroid plexus epithelial cells. The endothelial cells of the BBB are connected by a intricate network of tight junctions, which inhibits the free paracellular diffusion of water-soluble molecules. Combined with the absence of fenestrations and a low pinocytotic activity, these morphological peculiarities establish the physical permeability barrier of the BBB. At the same time, expression of a variety of transporters and enzymes that control the transport of nutrients into the CNS and of toxic metabolites out of the CNS establishes a metabolic BBB (1). Thus, morphology, biochemistry, and function make these brain microvascular endothelial cells unique and distinguishable from all other endothelial cells in the body.

Similar to the endothelial barrier, the morphological correlate of the BCSFB is found at the level of unique apical tight junctions between the choroid plexus epithelial cells inhibiting paracellular diffusion of water-soluble molecules across this barrier (2). The brain barriers also establish an interface between the peripheral immune system and the CNS. Immune cell migration into the CNS is rigorously controlled by mechanisms that operate at the level of the BBB and the BCSFB (3). During physiological conditions, migration of circulating immune cells into the CNS is very low and limited to specific immune cell subsets of the adaptive immune system and probably APCs, which maintain CNS immunosurveillance (3). In the event of CNS pathology, however, barrier characteristics of the blood–CNS barriers are often altered, leading to the recruitment of high numbers of inflammatory cells into the CNS. Although the molecular mechanisms involved in leukocyte migration across epithelial barriers are not well understood, extravasation of circulating leukocytes across a vascular wall has been studied in detail and requires the sequential interaction of different adhesion and signaling molecules on the leukocyte and their ligands on the endothelial surface (summarized in Ref. 4). The initial leukocyte interaction with the vascular wall leads to leukocyte rolling along the endothelial surface that greatly reduces the velocity of the leukocyte in the bloodstream. Reduced velocity allows the leukocyte to recognize chemokines on the endothelial surface, which trigger the G-protein–dependent activation of leukocyte integrins on the cell surface. Binding of activated integrins to their endothelial

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Abbreviations used in this article: BBB, blood–brain barrier; BCSFB, blood–cerebrospinal fluid barrier; FOV, field of view; GPCR, G-protein-coupled receptor; IF, immunofluorescence; JAM-A, junctional adhesion molecule-A; pMBMEC, primary mouse brain microvascular endothelial cell; PTX, pertussis toxin; PTX-B, pertussis toxin-B oligomer; RT, room temperature; VE-CadGFP-pMBMEC, primary mouse brain microvascular endothelial cell from mice expressing a C-terminal GFP fusion protein of VE-cadherin; wt, wild-type; xFMI, x forward migration index.

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ligands from the Ig superfamily mediates the arrest and subsequent crawling of the leukocyte on the endothelium and finally leukocyte diapedesis across the endothelial monolayer. Leukocyte diapedesis can take place through the endothelial junctions via a paracellular pathway or through the endothelial cell via a transcellular pathway (5).

The greatest body of information concerning the molecular mechanisms involved in the migration of immune cells into the CNS is available for the multistep extravasation of ependymaligenic CD4+ T lymphocytes across the BBB in chronic inflammatory situations such as experimental autoimmune encephalomyelitis, an animal model for multiple sclerosis (6). Knowledge of the molecular mechanisms mediating T cell migration across the inflamed BBB during experimental autoimmune encephalomyelitis has been translated into the clinic, where therapeutic targeting of α4β1 integrins mediating T cell arrest on the inflamed BBB (7) has proven beneficial for the treatment of relapsing-remitting multiple sclerosis (8, 9).

In contrast to other organs, the brain has proven to be quite unique in its resistance to neutrophil entry under inflammatory conditions (e.g., mimicked by intrathecal cytokine injection) (10, 11) or during ischemic stroke (12). Nevertheless, neutrophils accumulate in leptomeningeal spaces in systemic inflammatory conditions such as sepsis or in acute inflammatory states during bacterial meningitis or animal models of those and thus must have previously crossed the BBB or BSCFB (13, 14). Once neutrophils have crossed the brain barriers, they might significantly contribute to CNS injury by releasing their soluble mediators and their cytotoxic cargo (15). An involvement of Mac-1 over LFA-1 (20, 21) was found to be a prerequisite for transcellular neutrophil diapedesis. The two integrins αMβ2 and α4β1 have been shown to mediate neutrophil migration across the inflamed brain barriers under these conditions might allow developing therapies that specifically block or reduce neutrophil entry into the CNS with the aim to avoid neutrophil-mediated tissue injury.

There is a large body of literature comprising elegant in vivo live-cell imaging studies as well as detailed in vitro investigations that have allowed us to define the molecular mechanisms involved in the multistep neutrophil extravasation across the vascular wall in peripheral tissues (summarized in Ref. 17). These studies have shown that under inflammatory conditions, neutrophils arrest via interaction of their β2 integrins LFA-1 (αLβ2L integrin; CD11a/CD18) and Mac-1 (αMβ2R integrin; CD11b/CD18) on endothelial ICAM-1 (18, 19) and subsequently crawl on the inflamed endothelium by an involvement of Mac-1 over LFA-1 (20, 21). Leukocyte crawling allows the neutrophils to rapidly cross the endothelial wall via a suitable site mostly through the endothelial junctions and more rarely through the endothelial cell body (reviewed in Ref. 5). The molecules that have been shown to mediate neutrophil diapedesis across the inflamed endothelium in these peripheral vascular beds include PECAM-1, CD99, ICAM-1, ICAM-2, and junctional adhesion molecule A (JAM-A) (as summarized in Refs. 17, 22). Functional absence of β2 integrins completely abrogates extravasation of neutrophils in peripheral organs in vivo (23) and in humans (24).

A number of studies have recently addressed the molecular mechanisms involved in neutrophil migration across the BCSFB in the context of meningitis (14). In contrast, little is known about the signals that regulate neutrophil migration across the BBB in acute neuroinflammation. Studies performed to specifically address this issue are sparse (25), and although unique mechanisms of neutrophil extravasation across of the BBB have already been suggested (26), consideration of flow has been omitted in these previous studies.

In this study, we investigated selectin-independent neutrophil interactions with the BBB and therefore focused on investigating the role of neutrophil β2 integrins and their endothelial ligands ICAM-1, ICAM-2, and JAM-A in the multistep neutrophil extravasation across the inflamed BBB under physiological flow in vitro. To this end, we employed an in vitro model of the BBB, in which primary mouse brain microvascular endothelial cells (pMBMECs) that maintain physiological barrier characteristics (27), are placed in a flow chamber. Combined with high-magnification live-cell imaging, this model has proven to reliably mimic the dynamic behavior and the molecular mechanisms involved in the interaction of T cells or neutrophils with the BBB in neuroinflammatory disorders (12, 28, 29). Live-cell imaging of the interactions of neutrophils isolated from wild-type (wt), CD11a−/−, CD11b−/−, or CD18−/− mice with wt, JAM-A−/−, ICAM-1−/−, ICAM-2−/−, or ICAM-1null/ICAM-2−/− pMBMECs under physiological flow in vitro allowed us to distinguish the involvement of the different adhesion molecules in neutrophil arrest, polarization, crawling, and diapedesis. LFA-1 was found to be the prevailing ligand for endothelial ICAM-1 in mediating shear resistant arrest, whereas Mac-1 over LFA-1–mediated neutrophil polarization on the BBB in vitro. A contribution of both β2 integrins and endothelial ICAM-1 and ICAM-2 to neutrophil crawling was suggested by the observation that complete abrogation of neutrophil crawling could only be observed in the combined absence of either endothelial ICAM-1 and ICAM-2 or of all β2 integrins on neutrophils. Interestingly, we found no significant involvement of endothelial JAM-A or for the αL-integrin ligand VCAM-1 in neutrophil interaction with the inflamed BBB in vitro. Crawling neutrophils crossed the BBB endothelium via both the paracellular or the transcellular pathway, whereas diapedesis of stationary neutrophils occurred exclusively via the paracellular route. Thus, β2 integrin–mediated neutrophil crawling on endothelial ICAM-1 and ICAM-2 was found to be a prerequisite for transcellular neutrophil diapedesis across the inflamed BBB.

**Materials and Methods**

**Mice**

Gene-targeted mice were backcrossed to the C57BL/6 background for at least eight generations. ICAM-1−/− deficient mice generated by the deletion of the entire coding region of the ICAM-1 gene ([Itcamim1ltm3Jbgltm1ltb](10)) were kindly provided by D.C. Bullard and S.R. Barum (Birmingham, AL). To avoid confusion with other ICAM-1 mutant mice that express alternatively spliced isoforms of ICAM-1 (31), these mice have been referred to as ICAM-1null (32). This nomenclature is therefore maintained in the current study. ICAM-2−/− mice ([Itcam2ltsltm2ltb](11) referred to as ICAM-2−/−) were described before (33). CD11a−/− mice ([Itgaltm1bilo](12), referred to as CD11a−/− (34) and CD11b−/− mice ([Igfltm1aDib](13), referred to as CD11b−/−) (35) were purchased from The Jackson Laboratory (Bar Harbor, ME). JAM-A−/− mice ([F111ltm1.1Dib](14), referred to as JAM-A−/−) (36) were obtained from E. Dejana (Milan, Italy). Complete CD18−/− mice ([Igb2ltsl1Dib](15), referred to as CD18−/−) (37) and the knockout VE-Cadherin-GFP mice (38) were provided by D. Vestweber (Münster, Germany). All mice were bred in individually ventilated cages in our animal facility and kept under specific pathogen-free conditions. Animal procedures were performed in accordance with the Swiss legislation on the protection of animals and approved by the veterinary office of the Kanton of Bern.

**Abs and reagents**

The following hybridoma culture supernatants were used as a primary Abs: rat anti-mouse VE-cadherin (1D14.1) (described in Ref. 39) and rat anti-mouse ICAM-1 (25ZC7) were obtained from D. Vestweber; rat anti-mouse JAM-A (BV12) was obtained from E. Dejana; rat anti-mouse PECAM-1 (Mec13.3), rat anti-mouse ICAM-2 (3C4), rat anti-mouse CD45 (M1/9), rat anti-mouse CD11a (FD441.8), rat anti-mouse CD11b (M1/70), and rat anti-human CD44 (9B5; used as isotype control) were all purchased from American Type Culture Collection. Rat anti-mouse VCAM-1 (6C7.1), rat anti-mouse endothlin (M17/8), and rat anti-mouse CD49d (PS/2, 3-5, and R1.2) have been described in great detail previously (40). Additional primary Abs used were rabbit anti-mouse claudin-5 (341600; Invitrogen, Carlsbad, CA; 1:100), rabbit anti-mouse ZO-1 (617300; Invitrogen, 1:100), mouse ICAM-1 (25ZC7), rat anti-mouse ICAM-2 (3C4), rat anti-mouse CD45 (M1/9), rat anti-mouse CD11a (FD441.8), rat anti-mouse CD11b (M1/70), and rat anti-human CD44 (9B5; used as isotype control) were all purchased from American Type Culture Collection. Rat anti-mouse VCAM-1 (6C7.1), rat anti-mouse endothlin (M17/8), and rat anti-mouse CD49d (PS/2, 3-5, and R1.2) have been described in great detail previously (40). Additional primary Abs used were rabbit anti-mouse claudin-5 (341600; Invitrogen, Carlsbad, CA; 1:100), rabbit anti-mouse ZO-1 (617300; Invitrogen, 1:100),
rabbit anti-mouse occludin (71100; Invitrogen, 1:50), and rat anti-mouse CD49d (clone SG31, 28270/D; BD Pharmingen, San Diego, CA; 10 μg/ml). Secondary Abs for immunofluorescence (IF) were Alexa Fluor 488–conjugated goat anti-rabbit IgG and Alexa Fluor 488–conjugated goat anti-rat IgG (Molecular Probes, Eugene, OR; 1:1000), for flow cytometry staining, PE-conjugated goat F(ab’2) anti-rat IgG (Molecular Probes, 10 μg/ml) was used, and for infrared imaging, Alexa Fluor 680–conjugated goat anti-rat IgG (Molecular Probes; 1:5000) was used. LPS (Escherichia coli 055:BS) was purchased from Sigma-Aldrich (St. Louis, MO). Pertussis toxin (PTX) was obtained from List Biological Laboratories (Campbell, CA). PTX-B oligomer (PTX-B) was purchased from Calbiochem (San Diego, CA). PTX-B lacks G-i-inhibitory activity, but confers cell-surface binding specificity, and therefore, it was used as control for PTX.

pMBMECs

pMBMECs were isolated from 6–9 wk-old mice exactly as described by us before (29, 41). pMBMECs were used for flow assays after 6 or 7 d in vitro.

Neutrophil isolation from mouse bone marrow

Neutrophils were freshly isolated from the bone marrow of 10–15 wk-old C57BL/6 mice. Femur and tibia were removed and placed in HBSS (Life Technologies-BRL, Invitrogen) on ice. The bone marrow was flushed with HBSS using a 10-ml syringe and 25-gauge needle, and a single-cell suspension was obtained by careful pipetting. After erythrocyte lysis in isotonic ammonium chloride (NH4Cl) buffer, the cell suspension was filtered through a 70-μm pore size nylon mesh cell strainer (BD Biosciences, Franklin Lakes, NJ). Progenitor cells with high density were separated by centrifugation over a Percoll gradient (65% (v/v) Percoll in HBSS at 1000 g) covered and again separated by a Percoll gradient of 62% (v/v) at 1000 g for 10 min. After centrifugation, the resulting cell pellet was collected and resuspended in DMEM (Life Technologies-BRL) to neutral pH.

Flow cytometry

High purity of neutrophils was confirmed by four-color FACS analysis using the following directly labeled Abs: PE-conjugated rat anti-mouse CD19 (6D5), allopbycynin-conjugated rat anti-mouse CD11b (M1/70), FITC-conjugated rat anti-mouse Ly6G (1A8), and PerCP-conjugated rat anti-mouse CD45 (30-F11) (all obtained from BioLegend, San Diego, CA). For single-color FACS analysis of the cell-surface expression of CD49d, CD45, CD11a, and CD11b, 5 × 105 cells/sample were washed twice with FACS buffer-BRL (Invitrogen) and resuspended in 250 μl of FACS buffer-BRL supplemented with 2 μg/ml Fcy III/II receptor blocking Ab (clone 2.4G2) for 10 min. Ab incubation was performed for 30 min with primary Abs and with the PE-conjugated secondary Ab at 4°C with washing steps in between. Cells were then fixed in 1% formaldehyde/PBS (pH 7.4), and flow cytometry was performed using an FACS Calibur and CellQuest (BD Biosciences) and FlowJo (Tree Star Ashland, OR) software for data analysis.

Immunochemistry

For IF staining, pMBMECs were seeded on eight-well Lab-Tek chamber slides (Nunc, Roskilde, Denmark). Cells were washed in TBS and fixed with 1% (v/v) paraformaldehyde for 15 min at RT or methanol for 30 s at 4°C. Cells were incubated in IF blocking buffer (5% skimmed milk [w/v], 0.3% Triton X-100 [v/v], and 0.04% [w/v] NaN3 in TBS) for 30 min at RT. Stained cells were mounted with Mowiol (Calbiochem), and images were obtained using a Nikon Digital Camera (DXM1200F or DS-RiI; Nikon Instruments, Melville, NY) equipped with a Nikon Digital Camera (DXM1200F or DS-RiI; Nikon Instruments). Images were processed with NIS Elements Version 3.2 software.

Results

Establishment of an in vitro model of the acutely inflamed BBB

To mimic acute inflammatory conditions allowing neutrophil migration across the BBB in vitro, we established an acutely inflamed BBB model by stimulating pMBMECs with 100 ng/ml LPS for 5 and 24 h. We first compared the cell-surface expression of the endothelial cell adhesion molecules, ICAM-1, ICAM-2, JAM-A, VCAM-1, and PECAM-1 (CD31) involved in neutrophil migration across inflamed vascular walls in peripheral tissues on resting and LPS-stimulated pMBMECs. Cell-surface expression of ICAM-1 was significantly upregulated 5 and 24 h after LPS stimulation, whereas that of VCAM-1 was only significantly increased after 24 h of LPS challenge. Surface expression of ICAM-2, JAM-A, or PECAM-1 including junctional localization of JAM-A and PECAM-1 remained unchanged upon LPS stimulation (Supplemental Fig. 1A, 1B). Furthermore, LPS exposure did not disturb barrier characteristics of pMBMECs, as junctional integrity (Supplemental Fig. 1C) and paracellular permeability of pMBMEC monolayers (Supplemental Fig. 1D) were not affected.

To define comparability of LPS-induced effects on pMBMECs in vitro with LPS-induced inflammation of the BBB in vivo, C57BL/6 mice were i.v. injected with 50 μg LPS, sacrificed 4 h later, and...
expression of endothelial cell adhesion molecules was investigated on frozen brain sections by immunohistochemistry (Supplemental Fig. 2). Although we found a markedly increased immunostaining for endothelial ICAM-1 on cortical brain microvessels of LPS-injected mice, low constitutive immunostaining for VCAM-1 remained unchanged. Similarly, intensity of immunostainings for endothelial ICAM-2, JAM-A, and PECAM-1 remained unchanged in brain cortical microvessels of LPS-injected mice when compared with controls (Supplemental Fig. 2). Taken together, these observations demonstrate that the in vitro BBB model used in this study mimics the LPS-induced cell adhesion molecule phenotype of the BBB in vivo and is therefore perfectly suited to model leukocyte trafficking across the BBB during acute inflammation.

**LPS-stimulated BBB endothelium support neutrophil arrest, crawling, and diapedesis**

To analyze the dynamic interactions of neutrophils with the LPS-stimulated BBB in vitro, we performed live-cell imaging in a flow chamber experimental setup. Nonstimulated pMBMECs did not support any adhesive neutrophil interactions under physiological flow. In contrast, neutrophils readily arrested on 5 h LPS-stimulated pMBMECs. After shear-resistant arrest, the majority of neutrophils (84.3 ± 7.3%) started to crawl on the pMBMECs surface, whereas the rest remained stationary (Fig. 1A, Supplemental Video 1). To characterize neutrophil behavior in a quantitative manner, we assigned neutrophils to different behavioral categories depending on their interactions on pMBMECs as observed in a frame-by-frame offline analysis of the time-lapse videos. In the group of neutrophils crawling on LPS-stimulated pMBMECs, we distinguished neutrophils that continuously crawled during the 30 min of recording (category: continuous crawling, 36.2 ± 13.9% of the total of all arrested neutrophils) from those that underwent diapedesis after crawling (crawling with diapedesis: 35.6 ± 8.4%) (Fig. 1B). A minority of neutrophils (6.7 ± 5.2%) abruptly stopped after crawling and remained stationary or even detached during the acquisition time. Within the smaller group of neutrophils remaining stationary from the beginning, we observed cells that remained stationary during the entire observation time (category: stationary, 6.8 ± 3.2%) and neutrophils that crossed the pMBMEC monolayer at the very same spot of their arrest (stationary with diapedesis, 11.1 ± 6.3%). A small percentage of stationary neutrophils eventually detached during the acquisition time (stationary with detachment, 2.6 ± 0.8%).

Next, we speculated that the crawling behavior of neutrophils might be predictive for their success in finding a site permissive for diapedesis across the LPS-stimulated pMBMEC monolayers. The crawling velocities of neutrophils that succeeded or failed to cross the pMBMEC monolayer during the observation time was indistinguishable with 9.30 ± 2.64 and 9.15 ± 2.59 μm/min, respectively (Fig. 1C). Also, both groups of neutrophils preferentially crawled in the direction of flow (Fig. 1D). Interestingly, though, neutrophils succeeding to cross the pMBMECs used more intricate crawling paths, as shown by the significantly lower xFMI of 0.56 ± 0.24 when compared with the xFMI of 0.62 ± 0.16 observed for the group of continuously crawling neutrophils (Fig. 1E).

**G-protein–coupled receptor signaling is involved in neutrophil arrest and essential for postarrest neutrophil interaction with the inflamed BBB endothelium**

To investigate if G-protein–coupled receptor (GPCR)–dependent integrin activation is involved in neutrophil interaction with LPS-stimulated pMBMECs, neutrophils were pretreated for 2 h with 1 μg/ml PTX inhibiting Gso signaling or with PTX-B as a control. PTX-pretreated neutrophils showed a significantly reduced ability to firmly arrest on the inflamed BBB endothelium under flow (Fig. 2A). Reduced arrest of PTX-pretreated neutrophils was followed by a reduced polarization (Fig. 2B). Interestingly, although PTX-B pretreatment had no effect on neutrophil arrest, it slightly reduced neutrophil polarization on pMBMECs. Reduced polarization in PTX-B–pretreated neutrophils did not affect subsequent crawling and diapedesis of neutrophils. In contrast, absence of GPCR-dependent signaling in PTX-pretreated neutrophils completely abrogated their ability to crawl on pMBMECs and to subsequently cross the endothelial monolayer (Fig. 2C). Rather, PTX-treated neutrophils remained stationary or detached and failed to cross the BBB endothelium during the observation time (Fig. 2D). The involvement of GPCR-dependent signaling in neutrophil arrest and polarization on the LPS-stimulated pMBMECs and its necessity for neutrophil crawling on and subsequent diapedesis across the brain endothelium suggested a critical role for protein dependent activation of neutrophil β2 integrins in all of these events.

**Endothelial ICAM-1 mediates shear resistant arrest and polarization of neutrophils on the inflamed BBB endothelium**

To determine the specific role of the different endothelial β2 integrin ligands in neutrophil interaction with the in vitro BBB under flow, we used pMBMECs isolated from wt, ICAM-1null/ICAM-2null, ICAM-1null/ICAM-2−/−, ICAM-1null/ICAM-2−/−, and JAM-A−/− C57BL/6 mice. Lack of endothelial ICAM-1 on ICAM-1null or on ICAM-1null/ICAM-2−/− pMBMECs (Supplemental Videos 2, 3) significantly reduced the number of neutrophils able to arrest and polarize on pMBMECs under flow (Fig. 3A, 3B). In contrast, although absence of endothelial ICAM-2 or JAM-A seemed to reduce neutrophil arrest and polarization on pMBMECs under flow, these differences failed to reach statistical significance when compared with neutrophil interaction with wt pMBMECs (Fig. 3A, 3B). Furthermore, whereas on wt pMBMECs neutrophils typically crawled at velocities ranging from 3.6 to 15.6 μm/min, on ICAM-1null and more pronounced on ICAM-1null/ICAM-2−/− pMBMECs, 25.4 ± 4.2 and 64.9 ± 9.9% of the arrested neutrophils, respectively, continued to interact with the pMBMECs at velocities ≥16 μm/min in the direction of flow (Fig. 3C, Supplemental Videos 2, 3). The average velocity of neutrophils in this category, which we designated “accelerated movement,” was 20.31 ± 5.97 μm/min on ICAM-1null and significantly reduced on ICAM-1null/ICAM-2−/− pMBMECs. Accelerated movement of neutrophils was accompanied by an increase of their xFMI (Fig. 3D, 3E), suggesting that in the absence of endothelial ICAM-1 and ICAM-2, the avidity of neutrophil interactions with pMBMECs is significantly reduced. These observations also point to an important role of endothelial ICAM-1 and ICAM-2 in mediating neutrophil crawling on the BBB endothelium under flow.

**Postarrest neutrophil crawling on the inflamed BBB is mediated by endothelial ICAM-1 and ICAM-2**

To define the role of endothelial ICAM-1 versus ICAM-2 in neutrophil crawling on pMBMECs and the relevance of neutrophil crawling for successful diapedesis across the BBB in vitro, we next evaluated postarrest neutrophil behavior on wt, ICAM-1null, ICAM-2−/−, ICAM-1null/ICAM-2−/−, and JAM-A−/− pMBMECs within the three behavioral categories: crawling, stationary, and accelerated movement (Fig. 4). Neutrophil crawling was significantly reduced on ICAM-1null but not on ICAM-2−/− or JAM-A−/− pMBMECs and completely abrogated on ICAM-1null/ICAM-2−/− pMBMECs, confirming a role of endothelial ICAM-1 over ICAM-2 in neutrophil crawling on the BBB in vitro (Fig. 4A). The number of neutrophils per field of view (FOV) remaining stationary after
shear resistant arrest was comparable on all pMBMECs (Fig. 4B), suggesting an ICAM-1– and ICAM-2–independent arrest mechanism for this group of neutrophils. Accelerated movement of neutrophils was only observed on ICAM-1 null and ICAM-1 null/ICAM-2 null pMBMECs with a significantly higher number of neutrophils showing accelerated movement on ICAM-1 null/ICAM-2 null pMBMECs compared with ICAM-1null pMBMECs (Fig. 4C). These observations further underline the partially redundant roles for endothelial ICAM-1 and ICAM-2 in mediating neutrophil crawling on the BBB endothelium.

Within all three behavioral categories, we observed neutrophils that crossed the pMBMEC monolayer, although in the group of neutrophils showing accelerated movement on ICAM-1 null or ICAM-1null/ICAM-2 null pMBMECs, events of diapedesis were in fact rare (Fig. 4C). In general, in the absence of endothelial ICAM-1 or ICAM-1 and ICAM-2 combined, but not of ICAM-2 or JAM-A, the number of neutrophils that was able to cross the pMBMEC monolayer was significantly reduced (Fig. 4D). Interestingly, reduced diapedesis of neutrophils across ICAM-1null and across ICAM-1null/ICAM-2null pMBMECs was only observed for the group of neutrophils that crawled prior to diapedesis (Fig. 4D). In contrast, the total number of neutrophils undergoing diapedesis was not significantly affected by the lack of endothelial ICAM-1 and ICAM-2, underlining that this process is independent of these Ig cell adhesion molecules and thus different from diapedesis of crawling neutrophils across the inflamed BBB.

FIGURE 1. Characterization of the dynamic adhesive interactions of neutrophils on LPS-stimulated brain endothelium under physiological flow. LPS-stimulated pMBMECs supported neutrophil arrest, crawling, and diapedesis. Neutrophils that arrested on the pMBMECs during the accumulation phase and resisted detachment for 1 min after flow enhancement were defined and counted as arrested neutrophils. (A) The two behavioral categories "crawling" and "stationary" were defined based on the postarrest behavior of neutrophils on pMBMECs; 84.3 ± 7.3% of firmly arrested neutrophils crawled and 15.8 ± 7.4% remained stationary on LPS-treated pMBMECs. (B) Characterization of postarrest dynamic neutrophil interactions with LPS-stimulated pMBMECs. Only neutrophils that remained within the FOV over the entire observation time were included into the analysis, and their total number was set to 100%. Each neutrophil was assigned to one behavioral category according to its behavior on pMBMECs as follows: crawling (left, y-axis): 1) continuous crawling: neutrophils that continuously crawled on the surface of pMBMECs during the 30 min recording; 2) crawling with diapedesis: neutrophils that crossed the pMBMEC monolayer after crawling; and 3) stop crawling: neutrophils that crawled and stopped during the acquisition time. Stationary neutrophils (right, y-axis): 1) stationary: neutrophils that remained stationary on the pMBMECs during the entire observation time; 2) stationary with diapedesis: neutrophils that crossed the pMBMEC monolayer at the same spot where they arrested; and 3) stationary with detachment: stationary neutrophils that eventually detached during the acquisition time. (C) Crawling velocities of neutrophils with and without diapedesis. Each point represents the velocity of a single neutrophil. (D) x/y diagrams of neutrophil tracks on LPS-treated pMBMECs of one representative video. For each track, the site of shear-resistant arrest was set to x/y = 0/0 of the diagram. End points of neutrophil tracks are indicated with dots. (E) Directionality of neutrophil crawling tracks expressed as xFMI toward the x-axis (xFMI = Dx/Dacc, where Dx is straight x-axis distance covered by the neutrophil, and Dacc is the accumulated total distance of neutrophil movement). With relation to the FOV, the direction of flow was along the x-axis from minus to plus. Data are mean ± SD. The data were obtained from a total of n = 723 cells analyzed in 10 movies from independent pMBMECs and neutrophil preparations. *p < 0.05 (unpaired t test).
Therefore, we next asked if the low constitutive expression levels of endothelial VCAM-1 observed on the BBB in vitro and in vivo (Supplemental Figs. 1, 2) would allow for α4 integrin-dependent adhesive neutrophil interactions on ICAM-1null/ICAM-2−/− pMBMECs. To this end, we first studied the cell-surface expression of α4 integrins on bone marrow–derived neutrophils by flow cytometry. Employing four different anti–α4 integrin Abs we detected, however, only low if any cell-surface expression levels for α4 integrins on neutrophils (Supplemental Fig. 3A). In contrast, cell-surface expression of the β2 integrins LFA-1 and Mac-1 could readily be detected. High cell-surface expression for α4 integrins was detected on CD44 effector T cells known to cross the BBB in an α4 integrin-dependent manner (Supplemental Fig. 3B). Blocking of endothelial VCAM-1 with an anti–VCAM-1 Ab on LPS-stimulated ICAM-1null/ICAM-2−/− pMBMECs neither reduced neutrophil arrest nor crawling (Supplemental Fig. 3C, 3D). Functional absence of VCAM-1 also did not affect the number of stationary neutrophils (Supplemental Fig. 3E) or of accelerated movement of neutrophils on LPS-stimulated ICAM-1null/ICAM-2−/− pMBMECs (Supplemental Fig. 3F). Thus, VCAM-1/α4 integrin–adhesive interactions do not significantly contribute to ICAM-1/ICAM-2–independent neutrophil interactions with the LPS-stimulated BBB.

**LFA-1 regulates neutrophil arrest and Mac-1 neutrophil polarization by interaction with endothelial ICAM-1 on the inflamed BBB under flow.**

Next, we asked which β2 integrin ligands for endothelial ICAM-1 and ICAM-2 contribute to neutrophil interaction with the inflamed BBB under flow. To this end, we isolated neutrophils from wt, CD11a−/−, CD11b−/−, or CD18null C57BL/6 mice and compared their interactions on wt (Supplemental Videos 4, 5) or ICAM-1null/ICAM-2−/− pMBMECs. The number of CD11a−/− or CD18null neutrophils able to arrest per FOV on LPS-stimulated pMBMECs under physiological flow was 27 ± 9 and 22 ± 8, respectively, and thus was significantly reduced compared with wt neutrophils (72 ± 23) (Fig. 5A). Although shear-resistant arrest of CD11b−/− neutrophils per FOV (54 ± 13) seemed also to be reduced, this difference was found to be not significant (Fig. 5A). Reduced arrest of CD11a−/− and CD18null neutrophils was indistinguishable from neutrophil arrest on ICAM-1null (33 ± 14) (Fig. 3A) or ICAM-1null/ICAM-2−/− pMBMECs (33 ± 17) (Figs. 3A, 5A), demonstrating that CD11a/CD18 (LFA-1) acts as a predominant ligand for endothelial ICAM-1 in mediating shear-resistant arrest of neutrophils on the BBB in vitro.

Subsequent polarization of neutrophils on LPS-treated pMBMECs was decreased in CD11a−/−, CD11b−/−, and CD18null neutrophils as observed for wt neutrophils in the absence of endothelial ICAM-1 and ICAM-2 (Figs. 3B, 5B). Interestingly, CD11b−/− and CD18null neutrophils showed a more severe polarization defect than CD11a−/− neutrophils, suggesting a predominant role for Mac-1 over LFA-1 in mediating neutrophil polarization on the LPS-stimulated BBB in vitro.

**LFA-1 and Mac-1 mediate neutrophil crawling on brain endothelial ICAM-1 and ICAM-2.**

We next investigated the contribution of LFA-1 and Mac-1 to ICAM-1− and ICAM-2–mediated neutrophil crawling on the inflamed BBB and found that both CD11a−/− (Supplemental Video 4) and CD11b−/− neutrophils were significantly impaired in their ability to crawl on LPS-stimulated pMBMECs (27.26 ± 20.66 and 48.20 ± 2.29% crawlers of total arrested neutrophils, respectively) when compared with wt neutrophils (78.53 ± 8.56% crawlers of total arrested neutrophils) (Fig. 5C). Thus, lack of Mac-1 on neutrophils did not reduce neutrophil crawling on LPS-stimulated pMBMECs to the same degree as lack of LFA-1. The observation that CD18null neutrophils completely failed to crawl on wt (1.26 ± 1.98% crawlers of total arrested neutrophils) (Supplemental Video 5) and on ICAM-1null/ICAM-2−/− pMBMECs (0% crawlers of total arrested neutrophils) confirmed the essential role for β2 integrins and a contribution of both LFA-1 and Mac-1 in mediating neutrophil crawling on pMBMECs.

Nevertheless, CD18null neutrophils were able to maintain adhesive interactions with LPS-stimulated pMBMECs under flow as shown by their ability to remain stationary on wt and on ICAM-1null/ICAM-2−/− pMBMECs (Fig. 5D). In addition, as already

**FIGURE 2.** PTX-treated neutrophils show reduced arrest, polarization, and crawling on inflamed BBB endothelium. (A) Number of untreated (control), PTX-B−, or PTX-pretreated neutrophils arrested on LPS-stimulated pMBMECs per FOV is shown. (B) Cell polarization was analyzed by measuring the neutrophil length from the leading edge to the trailing edge of crawling and stationary neutrophils in the FOV after 8 min of image acquisition. Effects of PTX on postarrest neutrophil interactions were further analyzed within the categories crawling (C) and stationary (D). (C) Untreated (control), PTX-B−, or PTX-treated crawling neutrophils were subdivided into the three categories: continuous crawling, crawling with diapedesis, or stop crawling. (D) Stationary neutrophils were classified as stationary, stationary with diapedesis, or stationary with detachment. A detailed description of the different categories is provided in Fig. 1. Bars represent mean ± SD of three movies from independent pMBMECs and neutrophil preparations (n = 3). *p < 0.05, **p < 0.001 versus control. $p < 0.05, $$p < 0.01 versus PTX-B (one-way ANOVA, followed by Tukey test).
observed for wt neutrophils interacting with ICAM-1null and ICAM-1null/ICAM-2−/− pMBMECs under flow (Fig. 4C), CD11a−/−, CD11b−/−, and CD18null neutrophils arrested on wt or ICAM-1null/ICAM-2−/− pMBMECs showed accelerated movement interacting with the pMBMECs at velocities ≥ 16 µm/min (Fig. 5E). In the absence of β2 integrins and their endothelial ligands ICAM-1 and ICAM-2 and thus lack of neutrophil crawling, neutrophils can maintain β2 integrin-independent adhesive interactions categorized as stationary or accelerated movement with LPS-stimulated brain endothelium under physiological flow. These latter interactions are, however, of lower avidity, as demonstrated by the increased number of neutrophils detaching from the inflamed ICAM-1−/− and ICAM-2−/− deficient BBB during the observation time.

Crawling neutrophils can cross the BBB endothelium using the paracellular or the transcellular pathway

As crawling neutrophils were most successful to migrate across LPS-stimulated pMBMECs under flow, we finally investigated if neutrophils cross the BBB via the paracellular or transcellular pathway.
route. To this end, we prepared pMBMECs from mice expressing a C-terminal GFP fusion protein of VE-cadherin (VE-CadGFP-pMBMECs), allowing for the visualization of the endothelial junctions and thus the pathway of neutrophil diapedesis across the pMBMECs by live-cell imaging. Interestingly, the majority of neutrophils that crawled on VE-CadGFP-pMBMECs were found to cross the brain endothelial monolayer via the paracellular pathway through the endothelial junction, a significant number of crawling neutrophils crossed the VE-CadGFP-pMBMEC monolayer via the transcellular pathway (Fig. 6A, Supplemental Videos 6, 7), with both pathways taking the same time duration (Fig. 6B).

All neutrophils observed to remain stationary had previously arrested right on top of an endothelial junction and crossed the pMBMEC monolayer exclusively via the paracellular route at this endothelial junction (Fig. 6A). Interestingly, the duration of the paracellular diapedesis of stationary neutrophils was significantly longer when compared with the duration of paracellular diapedesis of neutrophils that had crawled to the endothelial junction prior to diapedesis. In both cases, paracellular diapedesis of neutrophils across the BBB endothelium occurred preferentially through transcellular junctions (Fig. 6C).

In the absence of endothelial ICAM-1 or both ICAM-1 and ICAM-2, neutrophil crawling was abrogated (Fig. 4A), and the number of transcellular diapedesis events was significantly reduced compared with wt pMBMECs (Fig. 6D), thus indicating that ICAM-1– and ICAM-2–dependent crawling of neutrophils on pMBMECs is the prerequisite for transcellular diapedesis across the BBB endothelium.

Discussion

Using live-cell imaging, in this study, we demonstrate that upon stimulation with LPS, pMBMECs establish an in vitro BBB model (28), which supports the shear-resistant arrest, crawling, and diapedesis of neutrophils under physiological flow in vitro. This allowed us to investigate the molecules mediating the multistep neutrophil migration across the acutely inflamed BBB. Shear-resistant adhesive interactions of neutrophils with the BBB required GPCR-mediated activation of neutrophil $\beta_2$ integrins and engagement to endothelial ICAM-1. Although LFA-1 was the prevailing ligand for endothelial ICAM-1 in mediating shear-resistant arrest of neutrophils on the LPS-activated BBB in vitro, subsequent neutrophil polarization was mainly mediated by Mac-1. Crawling was mediated by LFA-1 and Mac-1 and by endothelial ICAM-1 and endothelial ICAM-2, as complete abrogation of neutrophil crawling on the inflamed BBB could only be observed in the combined absence of either both endothelial ICAM-1 and ICAM-2 or of all $\beta_2$ integrins on CD18null neutrophils. In the absence of crawling, few neutrophils maintained adhesive interactions with
the BBB endothelium by remaining either stationary or by displaying transient $\beta_2$ and $\alpha_4$ integrin–independent adhesive interactions along the direction of flow. The low avidity of the latter interactions categorized as accelerated movement was suggested by the fast displacement of the neutrophils at average velocities of $>20 \, \mu m/min$ along the direction of flow. In contrast, on wt pMBMECs, neutrophils typically crawled at velocities ranging from 3.6 to 15.6 $\mu m/min$. Interestingly, diapedesis of stationary neutrophils was feasible in the absence of endothelial ICAM-1, ICAM-2, and VCAM-1 and occurred exclusively via the paracellular pathway. Conversely, crawling of neutrophils on the BBB could lead to paracellular or transcellular diapedesis. Thus, $\beta_2$
integrin–mediated neutrophil crawling on endothelial ICAM-1 and ICAM-2 is a prerequisite for transcellular neutrophil diapedesis across the inflamed BBB.

In the current study, stimulating pMBMECs, which have previously been characterized as suitable in vitro BBB models (44), with LPS were sufficient to allow for adhesive interactions with neutrophils under shear flow. Nonstimulated pMBMECs did not engage adhesive interactions with neutrophils. Stimulation of pMBMECs with 100 ng/ml LPS upregulated expression of ICAM-1 already at 5 h of treatment, whereas expression and cellular localization of ICAM-2 and VCAM-1 and the junctional adhesion molecules PECAM-1 and JAM-A remained unchanged. These in vitro findings therefore allow mimicking of LPS-induced inflammation of the BBB in vivo, where we found LPS to increase immunostaining for endothelial ICAM-1 in cortical brain microvessels already at 4 h after i.v. injection. Endothelial immunostaining for ICAM-2, VCAM-1, JAM-A, and PECAM-1 on cortical brain microvessels remained comparable to that observed in the vehicle-treated control. Although LPS stimulation induced a proinflammatory phenotype of the BBB in vitro and in vivo, it did not alter barrier characteristics of the pMBMEC monolayers. LPS-induced impairment of barrier characteristics of pMBMECs could, however, be observed after stimulation with 10-fold higher concentration of LPS (1 μg/ml) (data not shown) in accordance with a previous study in which stimulation of rat brain microvascular endothelial cells with LPS (1 μg/ml) impaired barrier characteristics of the cell monolayer (45). In addition, prolonged exposure of brain endothelial cells to LPS over 12 h might lead to LPS-induced cell death (46). Thus, our experimental conditions rather mimic the acutely inflamed BBB in the absence of LPS-induced impairment of barrier characteristics.

In our study, we specifically focused on delineating the role of β2 integrins and their endothelial ligands ICAM-1, ICAM-2, and JAM-A in the multistep neutrophil migration across the acutely inflamed BBB. The importance of β2 integrins in the adhesion cascade mediating neutrophil extravasation into peripheral tissues has been broadly recognized due to patients suffering from leukocyte adhesion deficiency type I (24). In leukocyte adhesion deficiency type I patients, lack of expression of the common β2 integrin subunit (CD18) compromises cell-surface expression of all β2 integrins on immune cells, which leads to the complete inability of neutrophils to infiltrate sites of infection (24). Nevertheless, the role of β2 integrins in neutrophil migration across the BBB during acute neuroinflammation remains unknown. The observed resis-
tance of the brain to neutrophil entry in acute inflammation (10–12) combined with the knowledge that the BBB endothelium actively controls leukocyte entry into the brain (3) indicates that unique roles for β₂ integrins in-mediated neutrophil migration across the BBB may be expected.

Our present observations confirm a predominant role of β₂ integrins and their endothelial ligands in mediating neutrophil interactions with the acutely inflamed BBB.

LFA-1 was the predominant integrin that mediated shear-resistant arrest of neutrophils on the inflamed BBB by interacting with endothelial ICAM-1, whereas Mac-1 was found to be the major β₂ integrin involved in neutrophil polarization. We found that inhibition of GPCR signaling reduced neutrophil arrest and polarization and completely abrogated neutrophil crawling and diapedesis. As all of these events critically relied on the activity of neutrophil β₂ integrins, these observations underscore the involvement of chemokines or lipid mediators derived from the inflamed BBB endothelium, which, upon binding to their cognate GPCR on the neutrophils, trigger the GPCR-dependent activation of neutrophil β₂ integrins. Previous studies have demonstrated that combined presentation of rICAM-1 with bacterial chemotacticants or chemokines suffices to support neutrophil crawling under flow conditions in vitro (21, 47). The continuous involvement of such mediators in β₂ integrin–dependent neutrophil crawling on the BBB was supported by our observation that inhibition of Gαi–signaling with PTX completely abrogated neutrophil crawling on the inflamed BBB. Additional involvement of GPCR-dependent mechanisms such as cytoskeletal remodeling in neutrophil interaction with the BBB cannot, however, be excluded.

LFA-1 and Mac-1 are the two most abundant β₂ integrins on neutrophils. Whereas LFA-1 is constitutively expressed on the cell surface, cell-surface expression of Mac-1 can be further enhanced upon neutrophil activation by translocation of vesicle-stored Mac-1 to the neutrophil surface (48, 49). In our study, LFA-1 was found to be the prevailing integrin mediating shear-resistant arrest of neutrophils on the LPS-stimulated BBB. Our results are in accordance with previous observations in which LFA-1 was shown to mediate arrest of human neutrophils on inflamed HUVECs under flow in vitro (50) and of neutrophils in inflamed cremaster muscle venules in vivo (20). Thus, LFA-1 mediates shear-resistant arrest of neutrophils on inflamed endothelial cells in different vascular beds.

Subsequent neutrophil polarization on the inflamed BBB critically relied on Mac-1 over LFA-1 interaction with endothelial ICAM-1. A dominant role for Mac-1 in neutrophil polarization was described before when Mac-1 was shown to maintain neutrophil polarization by modulating myosin L-chain phosphorylation to suppress lateral cellular protrusions (51).

Crawling of neutrophils on the LPS-stimulated BBB depended on β₂ integrins and endothelial ICAM-1 and ICAM-2, as abrogation of neutrophil crawling was only observed in the complete absence of either all β₂ integrins or the combined absence of endothelial ICAM-1 and ICAM-2. These observations suggested an involvement of LFA-1 in mediating neutrophil crawling, which would be unique to the BBB, as previous studies demonstrated a dominant role of Mac-1 in regulating neutrophil crawling in inflamed mesenteric venules (52) or in inflamed cremaster muscle venules (20, 21, 53). Nevertheless, the ability of LFA-1 in mediating immune cell crawling has been shown for T cells crawling on immobilized ICAM-1 in vitro (54) and for T cells crawling on the inflamed BBB in vitro and in vivo, respectively (28, 55). In addition, LFA-1–mediated crawling of neutrophils has previously been described on immobilized ICAM-1 in vitro (47). As different proinflammatory stimuli were found to induce different molecular mechanisms of neutrophil interaction with vascular endothelium in vivo (56), the involvement of LFA-1 versus Mac-1 in mediating neutrophil crawling might depend on the inflammatory status of the respective endothelial cells and thus the density of the endothelial β₂ integrin ligands ICAM-1, ICAM-2, and JAM-A. In addition, a major contribution of Mac-1 in mediating neutrophil crawling might also depend on the activation status of the neutrophil itself (56). In vitro systems such as ours, in which neutrophils unlike the endothelial cells are not activated prior to the assay, might therefore underestimate a contribution of Mac-1 in neutrophil crawling.

Differential involvement of LFA-1 versus Mac-1 in neutrophil crawling might also influence the direction of neutrophil crawling. Whereas in our in vitro model of the acutely inflamed BBB, LFA-1–mediated crawling of neutrophils was mainly observed with the direction of the flow, with some neutrophils also moving perpendicular to the flow or in random orientations, in inflamed cremaster muscle venules, Mac-1–mediated neutrophil crawling was observed to preferentially occur perpendicular to the direction of blood flow (20, 57). In agreement with our findings, a recent study observed that LFA-1–mediated neutrophil crawling on surfaces uniformly coated with ICAM-1 and chemotactic factors was oriented with the direction of fluid flow (58). Interestingly, the same study demonstrated that T cells rapidly orient against the direction of flow during LFA-1–mediated crawling on these surfaces. Previous work from us and others demonstrated that LFA-1–mediated crawling of CD4⁺ effector/memory T cells on the inflamed BBB occurs preferentially against the direction of flow in vitro (28) and in vivo (55). In any case, the functional significance of the directionality of leukocyte crawling remains to be investigated as crawling with, perpendicular to, or against the direction of flow will eventually lead to diapedesis across the vascular wall.

Those neutrophils that arrested on top of an endothelial junction remained stationary in our assay system and eventually crossed the endothelial cell monolayer via paracellular diapedesis through this junction. Thus, neutrophil crawling obviously serves to find the endothelial junctions as sites for diapedesis, which is supported by our observation that the majority of crawling neutrophils crossed the brain endothelial monolayer by paracellular diapedesis through the endothelial junctions. Interestingly, paracellular diapedesis following neutrophil crawling was achieved in a significantly shorter time, implying that downstream signaling events triggered by endothelial ICAM-1 and ICAM-2 during neutrophil crawling resulted in junctional disengagement to allow for paracellular diapedesis of neutrophils. This view is supported by previous observations demonstrating that engagement of endothelial ICAM-1 on brain endothelial cells enhances tyrosine phosphorylation of the adherens junction protein VE-cadherin, a key regulator of the endothelial junction, involving a signaling cascade with sequential mobilization of calcium and the activation of the small GTPase RhoA, a key regulator of the actin cytoskeleton (59, 60). Indeed, endothelial ICAM-1–mediated signaling resulting in VE-cadherin phosphorylation was found to be necessary for successful neutrophil transmigration across non-CNS endothelium (61, 62). The unique barrier characteristics including highly complex tight junctions of brain endothelium might lead to an increase of events of transcellular diapedesis. In this study, we observed that >30% of crawling neutrophils crossed the brain endothelium via the transeellular pathway. Previous studies by us and others (63, 64) employing ICAM-1 mutants provided direct evidence that intracellular signaling triggered via the C-terminal domain of ICAM-1 in brain endothelium is essential for leukocyte diapedesis across brain endothelium in vitro. This implies that additional signaling events previously described to be initiated by endothelial ICAM-1...
in the current study. Nevertheless, activated neutrophils were shown to induce stimulation of the BBB seems to induce JAM-A–independent neutrophil extravasation across non-CNS endothelium (56, 66), little is known about outside-in signal activated by ICAM-2 clustering. Nevertheless, based on our present knowledge, it is tempting to speculate that ICAM-1 and ICAM-2 signaling during neutrophil crawling leads to the formation of focal adhesions that provide force-transduction platforms, leading to a structural remodeling of the brain endothelial cells allowing for transcellular diapedesis of the neutrophils.

Previous studies have described the existence of β2 integrin–independent events promoting neutrophil extravasation in peripheral vascular beds (summarized by Ref. 67). Although few in number in our present study, we did observe that some neutrophils still could arrest on the inflamed brain endothelium in the absence of all β2 integrins or the combined absence of endothelial ICAM-1 and ICAM-2. Neutrophils remained stationary on endothelial junctions, suggesting that paracellular diapedesis of neutrophils across LPS-stimulated brain endothelium is mediated by molecules other than β2 integrin ligands including PECAM-1 and CD99 as previously described (summarized in Ref. 5). In the absence of β2 integrins or their endothelial ligands, we additionally observed neutrophils engaging in short-lived adhesive interactions, which we grouped in the category accelerated movement, as this resulted in their fast displacement of the neutrophils along the direction of flow. The molecular nature of these low-avidity adhesive interactions remains to be defined.

α4 integrins have been shown to be central in mediating the migration of effector T cells across the BBB during autoimmune CNS inflammation (3) and have been suggested to mediate β2 integrin–independent neutrophil recruitment into the lung (68) or the heart (69). In agreement with previous reports (67), we found low if any cell-surface expression for α4 integrins on neutrophils in the current study. Nevertheless, activated neutrophils were shown to be able to adhere via α4β1 integrin to VCAM-1 under flow conditions in vitro (70). In this study, we failed to demonstrate any role for neutrophil α4 integrin/VCAM-1 interaction in mediating β2 integrin–independent neutrophil arrest, polarization, or crawling on LPS-stimulated pBMMECs. This suggests that low expression levels of α4 integrin on the neutrophils and of VCAM-1 on the acutely inflamed BBB do not suffice to support neutrophil interaction with the BBB.

Finally, we did not find any involvement of endothelial JAM-A in mediating neutrophil interaction with the acutely inflamed BBB. Besides mediating homophilic interactions with JAM-A on leukocytes, endothelial JAM-A can also engage in heterophilic interactions with LFA-1 (71). Endothelial JAM-A has been shown to mediate the migration of myeloid cells across a number of vascular beds in vitro and in vivo (summarized in Ref. 72). JAM-A seems to specifically mediate the paracellular diapedesis of neutrophils, as in the absence of JAM-A, neutrophils were found to be trapped in endothelial junctions of inflamed cremaster muscle venules depending on the inflammatory stimuli investigated (73).

Investigations of a role of endothelial JAM-A in mediating neutrophil diapedesis across the BBB in mouse models of meningitis produced apparently different results. Whereas functional blocking of JAM-A reduced neutrophil entry into the leptomeninges in a cytokine-induced model of meningitis (74), a similar approach failed to reduce neutrophil migration across the BBB in mouse models of virus- or bacterial-induced meningitis (75). Thus, LPS-induced stimulation of the BBB seems to induce JAM-A–independent neutrophil extravasation, as previously observed in cremaster venules upon stimulation with leukotrienes or platelet-activating factor (73).

Taken together, our study demonstrates that stimulation with LPS suffices to support neutrophil arrest and crawling on as well as diapedesis across an in vitro model of BBB under flow in vitro, suggesting that neutrophils might cross the BBB in the event of bacterial meningitis or systemic infections. LFA-1 was the prevailing ligand for endothelial ICAM-1 in mediating shear-resistant arrest of neutrophils on the BBB in vitro, whereas Mac-1 was found to be the main β2 integrin involved in neutrophil polarization. β2 integrin–mediated neutrophil crawling on endothelial ICAM-1 and ICAM-2 was a prerequisite for transcellular diapedesis of neutrophils across the BBB under flow. Interestingly, diapedesis of stationary neutrophils arrested on endothelial junctions occurred exclusively via the paracellular pathway and was found to be independent of β2 and α4 integrins and their endothelial ligands ICAM-1, ICAM-2, and VCAM-1, supporting the contribution of other molecules such as PECAM-1 and CD99 in this process. Lack of an involvement of JAM-A in neutrophil diapedesis across the LPS-stimulated BBB underlines that inflammatory stimuli are critical in determining the precise molecular mechanisms and cellular pathways of neutrophil diapedesis across the inflamed BBB.

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Disclosures

The authors have no financial conflicts of interest.

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