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Neutrophil Cerebrovascular Transmigration Triggers Rapid Neurotoxicity through Release of Proteases Associated with Decondensed DNA

Charlotte Allen,*1 Peter Thornton,*1,2 Adam Denes,* Barry W. McColl,*3 Adam Pierozynski,* Marc Monestier,+ Emmanuel Pinteaux,* Nancy J. Rothwell,* and Stuart M. Allan*

Cerebrovascular inflammation contributes to diverse CNS disorders through mechanisms that are incompletely understood. The recruitment of neutrophils to the brain can contribute to neurotoxicity, particularly during acute brain injuries, such as cerebral ischemia, trauma, and seizures. However, the regulatory and effector mechanisms that underlie neutrophil-mediated neurotoxicity are poorly understood. In this study, we show that mouse neutrophils are not inherently toxic to neurons but that transendothelial migration across IL-1β-stimulated brain endothelium triggers neutrophils to acquire a neurotoxic phenotype that causes the rapid death of cultured neurons. Neurotoxicity was induced by the addition of transmigrated neutrophils or conditioned medium, taken from transmigrated neutrophils, to neurons and was partially mediated by excitotoxic mechanisms and soluble proteins. Transmigrated neutrophils also released decondensed DNA associated with proteases, which are known as neutrophil extracellular traps. The blockade of histone–DNA complexes attenuated transmigrated neutrophil-induced neuronal death, whereas the inhibition of key neutrophil proteases in the presence of transmigrated neutrophils rescued neuronal viability. We also show that neutrophil recruitment in the brain is IL-1β dependent, and release of proteases and decondensed DNA from recruited neutrophils in the brain occurs in several in vivo experimental models of neuroinflammation. These data reveal new regulatory and effector mechanisms of neutrophil-mediated neurotoxicity (i.e., the release of proteases and decondensed DNA triggered by phenotypic transformation during cerebrovascular transmigration). Such mechanisms have important implications for neuroinflammatory disorders, notably in the development of antileukocyte therapies. The Journal of Immunology, 2012, 189: 000–000.

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It is not known whether cerebrovascular extravasation causes neutrophils to acquire a neurotoxic phenotype and, if so, whether it happens rapidly enough to contribute to acute brain injury. Therefore, the aim of this study was to test the hypothesis that IL-1–induced cerebrovascular transmigration triggers neutrophils to acquire a neurotoxic phenotype. We show in this study that transendothelial migration of neutrophils across cerebral endothelium critically alters neutrophil phenotype to a neurotoxic state and that neurotoxicity of transmigrated neutrophils is mediated via rapid release of a mixture of active proteases associated with decondensed DNA, referred to as neutrophil extracellular traps (NETs). NETs contribute to the defense against extracellular bacteria (16), but actions in the brain have not been described. Collectively, our data identify novel trigger and effector mechanisms of neutrophil-mediated neurotoxicity and highlight how a key neutrophil antimicrobrial strategy can also be damaging to host tissue.

**Materials and Methods**

**Animals**

Wild-type (WT) and IL-1α/β deficient (IL-1αβ−/−) mice, all on a C57BL/6 background, were bred in-house and maintained on a 12-h light/dark cycle. Sprague Dawley rats were purchased from Charles River (Margate, U.K.). Animal studies were performed under United Kingdom Home Office personal and project licenses, and protocols adhered to the Animals (Scientific Procedures) Act (1986).

**Focal cerebral ischemia and other models of neuroinflammation**

Focal cerebral ischemia was induced by transient (60 min) middle cerebral artery occlusion (MCAo), as described previously (4, 17). After MCAo, mice were subjected to 8 or 24 h of reperfusion. Some animals were injected i.p. with 0.05 IU vehicle or rat IL-1β (National Institute for Biological Standards and Controls, Potters Bar, U.K.) 50 min before undergoing MCAo, as described previously (12). LPS (4 μg; Sigma, Gillingham, U.K.) was injected into the striatum of mice to induce parenchymal inflammation. Intrastriatal coadministration of α-aminoadipic-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA; 15 nM/ml; Tocris, Bristol, U.K.) and human IL-1β (5 ng/μl; National Institute for Biological Standards and Controls) (2-min infusion at 0.5 μl/min) was carried out, as described previously, to induce striatal and cortical damage in rats (18–20).

**Primary mouse brain endothelial cell culture**

Primary cultures of mouse brain endothelial cells (MBECs) were prepared from the brains of 8–12-wk-old C57BL/6 mice, as described previously (21), with the following modifications. Isolated brain vessels were resuspended in maintenance medium consisting of DMEM-F-12 (Invitrogen, Carlsbad, CA), supplemented with 10% FCS, 1% nonessential amino acids, 2 mM glutamine, 1 U/ml penicillin, and 100 μg/ml streptomycin. For neutrophil-migration assays, microvessels were plated onto murine collagen IV (50 μg/ml; BD Biosciences)-coated 24-well cluster Transwell inserts (6.5 mm diameter, 3.0 μm pore; Corning, Tewksbury, MA). Puromycin (3 μg/ml) was added to the media for 3 d in vitro. Cultures were grown at 37°C in a 5% CO2 humidified atmosphere and were used after 14 d in vitro, once cells were confluent. Cultures were 95.8 ± 1.6% positive for PECAM-1 and expressed endothelial markers von Willebrand factor and zona occludens-1 (data not shown).

**bEnd.5 MBEC line cultures**

The bEnd.5 MBEC line, which closely resembles primary brain endothelium in culture (4), was purchased from the Health Protection Agency Culture Collections (Salisbury, U.K.). Cells were grown in DMEM (high glucose, 4.5 g/l; Invitrogen), supplemented with 10% FCS, 1% nonessential amino acid, 2 mM glutamine, 1 U/ml penicillin, and 100 μg/ml streptomycin.

**Primary cortical neuronal cell cultures**

Primary cortical neuronal cell cultures were prepared from embryonic (day-15) mice, as described previously (7). Cells were seeded at 180,000 cells/cm² and grown in Neurobasal Medium (Invitrogen), 2% B27 minus antioxidants (Invitrogen), 5% plasma-derived serum, 2 mM glutamine, 1 U/ml penicillin, and 100 μg/ml streptomycin. Neurons were used between 10 and 13 d in vitro. Cultures were, on average, 98% pure neurons (data not shown).

**Collection of naive neutrophils**

Freshly isolated neutrophils (termed naive neutrophils) were collected from 8–12-wk-old mice euthanized by CO2 inhalation. Femurs and tibias were removed, and bone marrow was flushed using a 25G needle with 1–2 ml neutrophil buffer (PBS without calcium or magnesium with 0.1% low endotoxin BSA [w/v] and 1 mM EDTA; Sigma). RBCs were lysed with 0.2% (w/v) NaCl, and osmolarity was restored by the addition of 1.2% (w/v) NaCl. Ly6G+ neutrophils were separated immunomagnetically by passing the previously labeled cell suspension through an LS column and magnet (Miltenyi Biotec, Bisley, U.K.). The column was removed from the magnet, and the eluted cells were washed and resuspended in serum-free neurobasal medium at 4 × 10⁶ cells/ml. The average purity of neutrophil preparations was 96%, as identified by flow cytometry (see below).

**Neutrophil transendothelial migration assay**

MBECs or bEnd.5 cells grown to confluence on Transwell inserts were pretreated for 4 h with IL-1/–β-stimulated brain endothelium grown on larger six-well–format Transwell inserts (4.7 cm² area/Transwell). For this purpose, and because of low yields of MBEC primary cultures, we used the bEnd.5 cell line to support neutrophil transmigration. For this transendothelial migration using larger Transwell inserts, a concentration of 10 ng/ml IL-1β for 4 h was used. This concentration induced a similar increase in neutrophil transmigration across bEnd.5 cells as observed with 100 ng/ml (Supplemental Fig. 1A). To induce the possibility of IL-1 carried over after activation. This allowed us to determine the effects of activated versus nonactivated endothelial-derived factors on nonmigrated neutrophil phenotypes. A purified neutrophil suspension containing 3.5 × 10⁵ cells was added to the luminal (top) compartment of each six-well Transwell. After the specified incubation period, the abluminal-transmigrated fraction of neutrophils (termed transmigrated neutrophils) was collected and centrifuged at 400 × g for 10 min, and cells were counted using a hemocytometer. Neutrophil transmigration was expressed as the fold increase compared with vehicle-treated (control) cultures.

**Collection of transmigrated neutrophils**

To obtain transmigrated neutrophils in sufficient quantities to analyze their phenotypes, we collected neutrophils that had migrated across IL-1β–stimulated brain endothelium grown on larger six-well–format Transwell inserts (4.7 cm² area/Transwell). For this purpose, and because of low yields of MBEC primary cultures, we used the bEnd.5 cell line to support neutrophil transmigration. For this transendothelial migration using larger Transwell inserts, a concentration of 10 ng/ml IL-1β for 4 h was used. This concentration induced a similar increase in neutrophil transmigration across bEnd.5 cells as observed with 100 ng/ml (Supplemental Fig. 1A). To induce the possibility of IL-1 carried over after activation. This allowed us to determine the effects of activated versus nonactivated endothelial-derived factors on nonmigrated neutrophil phenotypes. A purified neutrophil suspension containing 3.5 × 10⁵ cells was added to the luminal (top) compartment of each six-well Transwell. After the specified incubation period, the abluminal-transmigrated fraction of neutrophils (termed transmigrated neutrophils) was collected and centrifuged at 400 × g for 10 min. For the direct addition of neutrophils to neural cultures, transmigrated neutrophils were collected from the abluminal compartments 4 h after application of naive neutrophils to the luminal compartment. All nonmigrated neutrophil controls were exposed to bEnd.5 cells, which had been also been treated previously with vehicle or IL-1β (10 ng/ml) for 4 h, in the abluminal compartment of the Transwell insert. Previous studies showed that >1 h is sufficient to allow neutrophils to respond to endothelial-derived factors (13). In addition, naive neutrophils were incubated for 4 h in the presence of conditioned medium obtained from activated endothelium, washed, and incubated for 4 h. For the addition of neutrophil-conditioned medium to neuronal cultures, transmigrated neutrophils were collected 20 h after the addition of naive neutrophils to the luminal compartment. This time point allowed collection of sufficient neutrophils for subsequent analysis.

To induce neutrophil transendothelial migration outside the brain in vivo, a model of thioglycollate-induced peritonitis was used. Thioglycollate medium (3%), aged for 1 mo, was injected into the peritoneal cavity, and neutrophils were recovered by peritoneal lavage 4 h after injection. Contaminating RBCs were lysed, and neutrophils were washed and resuspended in neurobasal medium for addition to neurons.

**Collection of neutrophil lysates and neutrophil-conditioned medium**

To determine the effects of neutrophil-secreted factor(s) on neurons, neutrophil-conditioned medium was collected by resuspending neutrophils in serum-free neurobasal medium, at 1.2 × 10⁶ cells/ml, and seeding into tissue culture plates for 3 h. Cells were collected and centrifuged at 400 × g for 10 min, and pellets were lysed in lysis buffer containing protease...
inhibitors. Supernatants were further centrifuged at 10,000 × g for 10 min. Lyssates and cleared supernatants were aliquoted and stored at −80°C.

Treatment of neurons with neutrophils or neutrophil-conditioned medium

Direct application of neutrophils onto primary neuronal cultures was performed by adding 10 µl age-matched naive neutrophils, nonnegated neutrophils (using nonactivated endothelium, activated endothelium, or neutrophils that were also incubated for 4 h in the presence of conditioned medium obtained from activated endothelium, washed, and incubated for 4 h), or transmigrated neutrophils (suppressed at 4 × 10^6 cells/ml) to neurons grown in 96-well plates, corresponding to 120,000 neutrophils/cm^2 of neurons. Neutrophil-conditioned medium was added to neuronal cultures at a 1:4 dilution (one quarter of neuronal media was removed and replaced with neutrophil-conditioned medium). Neurons were treated with 10 µM MK-801 (Tocris), 10 µM cathepsin-G inhibitor (Calbiochem, Watford, U.K.), 10 µM elastase IV inhibitor (Calbiochem), 20 µM aprotinin (Sigma), or 12.5 µM metalloproteinase-9 (MMP-9) inhibitor (SB3CT; Enzo, Exeter, U.K.) added 20 min prior to treatment with neutrophil-conditioned medium. Neutrophil-conditioned medium was also pretreated with washed trypsin–agarose beads (1 U, 30 min at 37°C, Sigma) or was heat inactivated for 30 min at 95°C prior to application to neurons. PL2-3 monoclonal anti-ab (anti-H2A-H2B-DNA) or an IgG2a isotype control (22, 23) was incubated (15 µg/ml) with prechilled neutrophil-conditioned medium for 10 min before addition to neurons. Neutrophil-conditioned medium was also pretreated or not with DNase I (Invitrogen), at a concentration of 30 U/ml for 30 min at 37°C, before being added to neuronal cultures (as described above) for additional incubation lasting 24 h.

Live cell imaging

Neurons were loaded with 1 µM CellTracker Red (Invitrogen) for 30 min and washed into imaging buffer: 121 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl2, 1.8 mM CaCl2, 6 mM NaHCO3, 5.5 mM glucose, 25 mM HEPES (pH 7.3). Neurons were imaged every 30 s in the absence or presence of transmigrated neutrophils (neutrophils added to neuronal cultures at 120,000 cells/cm^2) on a transmitted light channel and on an Alexa Fluor 568–conjugated secondary Ab (1:1000 in PBS; Invitrogen) and mounted with ProLong Gold, with or without DAPI counterstain. Wide-field fluorescence images were collected on an Olympus BX51 upright microscope and captured using MetaVue software (Molecular Devices, Uckfield, U.K.). Z-stacked images were acquired on a Delta Vision (Applied Precision, WA) restoration microscope objective.

Immunohistochemistry

Neurons cultured on poly-L-lysine–coated 12-mm glass coverslips were fixed in 4% paraformaldehyde/4% sucrose (w/v), permeabilized with 0.1% (w/v) Triton X-100 in PBS, quenched with 0.25% (w/v) NH4Cl, and blocked with 5% (w/v) BSA in PBS. Cultures were immunostained with a combination of the following Abs: neurofilament (1:500; Millipore, Watford, U.K.), neuronal nucleus (1:200, Chemicon, Watford, U.K.), neutrophil elastase (1:500; Abcam), PL2-3 (1:500; anti–H2A-H2B-DNA complex), and SJC (1:5000). Immunodetection was performed with Alexa Fluor-conjugated secondary Abs (1:100; Invitrogen), and cells were mounted with ProLong Gold, with or without DAPI counterstain. Wide-field fluorescence images were collected on an Olympus BX51 upright microscope and captured using MetaVue software (Molecular Devices, Uckfield, U.K.). Z-stacked images were acquired on a Delta Vision (Applied Precision, WA) restoration microscope objective.

Immunocytochemistry

Transmigrated neutrophils were added to neuronal cultures, as described above, and fixed with 2.5% (w/v) glutaraldehyde (Sigma). The fixed cells then underwent three 5-min washes with 0.1 M PBS (Sigma). After the final wash, 1% (v/v) osmium tetroxide–Sigma (0.1 M PBS was added to the cells for a minimum of 1 h at 4°C. The cells were rinsed with 0.1 M PBS and dehydrated with a 15-min incubation in increasing alcohol solutions (70% and 95%) and then three additional incubations in 100% alcohol for 10 min. The tissue was dried to the critical drying point and then mounted for 3 min of sputter coating. The samples were viewed using the Gatan 3View System (Gatan, Abingdon, U.K.).

Flow cytometry

Cells were fixed in FACS/FIX buffer (1% [w/v] PFA and 0.1% [w/v] low-endotoxin BSA [Sigma]) for 15 min at room temperature. Cells were washed with FACS buffer (0.1% BSA [w/v] PBS) and pelleted at 400 × g for 5 min. Neutrophils were incubated with anti-CD16/CD32 for 30 min at 4°C (1:200 in FACS buffer; BD Biosciences) and then washed and pelleted, as described before. Staining was carried out with anti–Ly6G-allophycocyanin (1:200; eBiosciences, Hatfield, U.K.), anti–CD11b-Alexa Fluor 488-conjugated Ab (1:100; Serotec, Oxford, U.K.), or isotype control for 30 min at 4°C before final wash and centrifugation. Neutrophils were identified by flow cytometry. Ninety-six percent of the purified, naive neutrophil population was CD11b/Ly6G+.

For the staining of intracellular phosphorylated proteins, neutrophils were labeled with Ly6G, as before, but then permeabilized with PerFix (BD Biosciences) for 30 min. Cells were washed and collected by centrifugation at 400 × g for 5 min and incubated with anti-p–NF-κB–PE, anti–p-p38–PE, or anti–p-Akt–PE (1:25 in FACS buffer; eBiosciences) for 30 min at 4°C in the dark. After a final wash and centrifugation, the cells were resuspended in FACS/FIX buffer and analyzed by a Cyan ADP (Beckman Coulter, High Wycombe, U.K.) flow cytometer.

Tissue homogenization

WT or IL-10−/− mice were anesthetized 8 h after MCAo and perfused with 0.9% saline, and brain samples were rapidly removed and frozen on dry ice. Frozen cortical samples were homogenized on ice in buffer (50 mM Tris-HCl [pH 7.6], 150 mM NaCl, 5 mM CaCl2, 0.02% Na3VO4, 1% Triton X-100) and centrifuged (15,000 × g, 15 min, 4°C). The resulting supernatant was determined in supernatants by bicinchoninic acid protein assay (detection range: 800–1.55 µg/ml; Pierce, Cramlington, U.K.).

ELISA

ICAM-1 and VCAM-1 concentrations in cortical homogenates were quantified by ELISA, according to the manufacturer’s instructions (R&D Systems). Samples were read at 450/570 nm on a plate reader (Biotek, Bedford, U.K.), with a detection range of 8 ng/ml–13.25 pg/ml and intra-assay variation < 3%. Data obtained were analyzed using GraphPad Prism 5.0 software (GraphPad, CA).

Because of the high sensitivity and specificity of PL2-3 Ab (22), a specific histone-DNA complexes ELISA was developed in-house. PL2-3 Ab was used to detect the H2A-H2B-DNA complex immobilized from neutrophil-conditioned medium samples on Nunc (Sigma) microwells. Following incubation with biotinylated horse-anti-mouse Ab (Vector Laboratories) and streptavidin–HRP (R&D Systems), the reaction was developed with tetramethylbenzidine substrate solution (BD Biosciences). Samples were read at 450/570 nm on a Biotek plate reader (Biotek, Potton, U.K.).
Cytometric bead array
Lysates of naive neutrophils, nonmigrated neutrophils, and transmigrated neutrophils were assayed by Cytometric Bead Array (CBA) analysis (BD Biosciences) for the following 14 inflammatory mediators: MIP-1α, TNF-α, RANTES (CCL5), MCP, CXCL1, IL-6, IL-1α, IL-1β, IL-17, IL-10, IFN-γ, G-CSF, L-selectin, and E-selectin.

Identification of nuclear and mitochondrial DNA by PCR
Neutrophil-conditioned medium prepared from naive or transmigrated neutrophils was assayed for the presence of mitochondrial or nuclear DNA as follows: DNA was precipitated from a fixed volume of conditioned medium using sodium acetate/ethanol protocol before resuspension in 10 μl RNase-free distilled water. To confirm the presence of mitochondrial or nuclear DNA, sections of DNA within unique mitochondrial or nuclear genes were targeted by PCR using specific primers (sequence available on request) as follows. Because a fixed volume of neutrophil-conditioned medium was used for PCR reaction, a fixed volume of 1 μl was used for each PCR reaction. Each PCR reaction contained 1 mm MgCl2, 1X reaction buffer, 1.2 mM 2- deoxyinosine triphosphate, 1 pmol forward and reverse primer, and 2.5 U Taq polymerase (all reagents were from Bioline, London, U.K.) for 5 min at 94°C; 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30 s at 72°C, 5 min at 72°C and then a final step of 4°C for 15 min. Products were detected on a 1.5% (w/v) agarose gel containing 5 μg/ml ethidium bromide (Sigma) at 100 V for 45 min and visualized using ImageQuant LAS 400 (GE Healthcare, Chalfont St. Giles, U.K.).

Gel zymography
To determine the presence of active mature MMP-9, neutrophil-conditioned medium was collected from naive or transmigrated neutrophils and subjected to gelatin zymography, as described previously (21).

Western blot analysis
Total protein content of bEnd.5 lysates was determined using bicinchoninic acid assay. A fixed protein concentration of each sample was diluted in 5X dissociation buffer (200 mM Tris/HCl [pH 6.8], 10% SDS, 20% [v/v] glycerol, 10 mM DTT, 0.05% bromophenol blue). Samples were heated at 95°C for 5 min prior to being resolved by 12% SDS-PAGE. Proteins were then transferred onto a Hybond (polyvinylidene difluoride; GE Healthcare) membrane. ICAM-1 and VCAM-1 were then detected using ICAM-1 and VCAM-1 antibodies and processed using ImageQuant LAS 400 (GE Healthcare) for the following 14 inflammatory mediators: MIP-1, TNF-α, RANTES (CCL5), MCP, CXCL1, IL-6, IL-1α, IL-1β, IL-17, IL-10, IFN-γ, G-CSF, L-selectin, and E-selectin.

Trypan blue assay.
Nonmigrated or transmigrated neutrophils were collected 20 h after isolation or application to bEnd.5 cells and were incubated with 1:2 dilution of the vital dye trypan blue (Sigma); the percentage of trypan blue+ cells was determined.

Statistical analysis
Data are expressed as mean (± SEM) from a minimum of three independent experiments carried out on separate cultures. Depending on the number of groups within the data set, data were analyzed using either the Student t test (two groups) or one-way ANOVA with the Bonferroni post hoc test (three or more groups) using GraphPad Prism 5.0 (GraphPad). The p values < 0.05 were considered statistically significant.

Results
IL-1 mediates cerebrovascular activation and recruitment of neutrophils in vivo and induces cerebrovascular transmigration of neutrophils in vitro
We first used MCAo as a model to induce neuroinflammation and found that neutrophil migration in the brain was significantly reduced in IL-1α/β−/− mice compared with WT mice (Supplemental Fig. 1a–c). These observations were supported by the altered IL-1–dependent ICAM-1 and VCAM-1 levels in vivo (Supplemental Fig. 1e–g) and IL-1–treated bEnd.5 cells in vitro (Supplemental Fig. 1h). IL-1β induced the migration of neutrophils (CD11b/Ly6G+; Supplemental Fig. 2a) across primary cultures of MBEcs and bEnd.5 cells (Fig. 1a) into the abluminal compartments of Transwell inserts. Treatment with vehicle had little effect on migration (<3%) across MBECs or bEnd.5 cells. Activation of MBEcs by IL-1β caused a 4.5-fold increase in neutrophil migration compared with the vehicle-treated control (Fig. 1a), which was blocked by IL-1 receptor antagonist (Supplemental Fig. 2b). A comparable increase was seen in bEnd.5 cultures treated with the same concentration of IL-1β (Fig. 1a).

Cerebrovascular transmigration induces a neurotoxic phenotype in neutrophils, leading to rapid neuronal death
Transmigrated neutrophils applied to neurons for 3 h induced significant loss of neuronal viability (Fig. 1b), which was comparable to the neurotoxicity induced by treatment with N-methyl-D-aspartate (NMDA; 600 μM) for 24 h (Fig. 1b). Real-time CellTracker monitoring of healthy neurons showed that transmigrated neutrophils induced rapid neuronal death (Fig. 1c1, 1cii). Neuronal cell bodies loaded with CellTracker Red and their processes were visualized every 30 s for 6 h, in the absence or the presence of transmigrated neutrophils (Supplemental Videos 2 and 1, respectively). Neurons exposed to transmigrated neutrophils swelled rapidly (within 180 min), rounded up, and lost CellTracker Red staining intensity (Fig. 1c1i, Supplemental Video 1). Loss of CellTracker Red labeling was not apparent over a similar time period in untreated neurons (Fig. 1c1i, Supplemental Video 2). Neuronal processes became bead-like and fragmented within 30 min of application of transmigrated neutrophils (Fig. 1c1ii, Supplemental Video 1). Addition of transmigrated neutrophils to neuronal cultures for 24 h induced a marked loss of neuronal cell bodies and processes, as identified by PGP 9.5 staining (Fig. 1dii).

Neurotoxicity induced by the application of transmigrated neutrophils was confirmed by LDH release: a 3-fold increase in neuronal death was observed after 24 h (Fig. 1e). Application of naive neutrophils or nonmigrated neutrophils, which had been incubated with nonactivated or IL-1β–activated brain endothelial cells or incubated with conditioned medium from activated endothelium after washing, did not significantly affect neuronal viability after 24 h of incubation (Fig. 1e). Cell death was not due to IL-1β being carried over, because naive neutrophils incubated with neurons in the presence of IL-1β did not induce a significant increase in cell death within the same time frame (Supplemental Fig. 2d). This increase in LDH release observed within neurons after exposure to transmigrated neutrophils was not due to compromised neutrophil viability, which was determined through trypan blue staining: the viability of transmigrated neutrophils 20 h after isolation was 40% less than that observed for the naive neutrophils (Supplemental Fig. 2e).

The neurotoxicity of transmigrated neutrophils was also conveyed through the release of soluble factors from the neutrophils, because conditioned medium from transmigrated neutrophils (applied at 1:4 dilution to the primary neuronal cultures) induced

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a 4-fold increase in neuronal death (overall 30–40% increase in neuronal LDH release) after 24 h. Conditioned medium from naive neutrophils or nonmigrated neutrophils induced a reduction in neuronal viability to a much smaller extent compared with that induced by the conditioned medium from transmigrated neutrophils (Fig. 1f), which was comparable to the neurotoxicity induced by NMDA (600 μM) after 24 h of incubation (red dashed line). An increase in neurotoxicity was also observed in and the presence of transmigrated neutrophils after migration across IL-1β–stimulated bEnd.5 (Supplemental Fig. 2f). Therefore, this rapid induction in neuronal death could be solely attributed to the transmigration of neutrophils across activated brain endothelium, because no increase was observed in any of the four nonmigrated control groups.

**FIGURE 1.** Neutrophils acquire a neurotoxic phenotype after transmigration across activated brain endothelium. (a) Transmigration of neutrophils across brain endothelium cells (bEnd.5) and primary MBECs treated with IL-1β (4 h) after 24 h. (b) Bright-field images of neurons exposed to Neurobasal Medium, nonmigrated neutrophils (NM PMN), transmigrated neutrophils (TM PMN) (120,000 cells/cm²), or 600 μM NMDA in the presence of MTT (5 μg/ml) for 24 h. Neuronal viability under these conditions is indicated by the presence or absence of MTT crystals. Scale bar, 300 μm. (c) Bright-field and CellTracker Red images of stained neurons (arrowheads) exposed to TM PMN for 0–180 min (Supplemental Video 1). Scale bar, 15 μm. (d) Bright-field and DAPI images of a neuronal process (arrows) exposed to TM PMN for 0–135 min. Scale bar, 5 μm. (e) Immunofluorescent images of neurons (PGP 9.5, green) exposed to naive PMN (i) or TM PMN (ii) (120,000 PMN/cm²) for 24 h. Scale bars, 30 μm. (f) Quantification of total LDH release from neurons 4 or 24 h after application of naive neutrophils (PMN), nonmigrated neutrophils that were incubated with nonactivated endothelial cells (NM–PMN), nonmigrated neutrophils that had been incubated with IL-1β–activated brain endothelial cells (NM+ PMN) or incubated with conditioned medium from activated endothelium after washing (NMTW+PMN), or transmigrated neutrophils (TM PMN) (120,000 cells/cm²). Red dashed line indicates neuronal death induced by 600 μM NMDA. Effect of conditioned medium (applied at 1:4 dilution) from naive PMN, NM–PMN, NM+ PMN, NMTW+ PMN, or TM PMN on neuronal viability, as assessed by LDH release after 24 h. Bars represent mean ± SEM for a minimum of three independent experiments carried out on separate cultures. Red dashed line indicates neuronal death induced by 600 μM NMDA. **p < 0.01, one-way ANOVA, with a Bonferroni post hoc test.
We also investigated whether transendothelial migration-induced neutrophil neurotoxicity is unique to activated brain endothelium. To this end, we used a thioglycollate-induced peritonitis model, which also allowed us to assess the role of in vivo neutrophil transmigration on neurotoxicity in vitro. Conditioned medium from neutrophils collected from the peritoneal cavity after thioglycollate injection was neurotoxic and led to increased LDH release and reduced MTT metabolism in neuronal cultures (Supplemental Fig. 2g, 2h).

Cerebrovascular transmigration profoundly alters neutrophil phenotype

The morphology of naive neutrophils and transmigrated neutrophils was determined by immunofluorescence. Compared with naive neutrophils, transmigrated neutrophils were devoid of lobed nuclei (Fig. 2bA, arrow) and displayed extracellular decondensed DNA (Fig. 2bB, arrow), which was associated with an increase in the abundance of elastase staining (Fig. 2bB, 2bC). Using immunofluorescence, the loss of neuronal MAP2 staining observed after 4 h of incubation with transmigrated neutrophils was extensive in comparison with the neurons incubated with the naive controls (Fig. 2c, 2d). In this instance, the neutrophils were also stained with CD45, as well as MMP-9, indicating the presence of the secondary and tertiary granules. A three-dimensional z-stack was produced from the wide-field fluorescent images, allowing visualization of the morphology of naive and transmigrated neutrophils in the presence of the neuronal cultures in a transverse cross section (Fig. 2cii, 2dii). The presence of the punctuate MMP-9 staining within the spherical naive neutrophils can be clearly seen, resting above the neuronal cultures (Fig. 2cii). This MMP-9 staining was lost in the transmigrated neutrophils, which, together with the altered shape and appearance, suggests their degranulation (Fig. 2dii).

We then determined the inflammatory phenotype of the transmigrated neutrophils in comparison with naive neutrophils by CBA analysis (Table 1). Transmigrated neutrophils had increased inflammatory profiles, with reduced cell-associated CD62L (L-selectin) indicative of activation-induced CD62L shedding, as well as significantly increased neutrophil CXCL1 and IL-6 expression. We also detected a significant increase (six-fold) in neutrophil cell-associated (but not secreted) inflammatory factors RANTES and MCP-1 (Table 1). These results show that cerebrovascular transmigration profoundly alters neutrophil viability, activation, and inflammatory phenotype.

Flow cytometric analysis demonstrated a rapid activation of transmigrated neutrophils after transmigration (Supplemental Fig. 3ai–iii). The percentage of transmigrated neutrophils staining positive for both p-p38 and p-Akt was significantly increased in comparison with the naive neutrophils, and a trend toward increased p–NF-κB was observed (Supplemental Fig. 3ai, 3aiii). Activation of the p–NF-κB, p38, and Akt pathways was shown to promote survival of activated neutrophils (24–26). These data are consistent with previous studies on noncerebral endothelium, demonstrating that neutrophil transmigration across endothelium prolongs neutrophil viability (27, 28).

Release of neutrophil proteases associated with decondensed DNA is linked with neuronal death in vitro

Following our previous observation of decondensed DNA within transmigrated neutrophils (Fig. 2bB), we hypothesized that the neurotoxicity of transmigrated neutrophils was mediated by histone–DNA complexes or neutrophil-derived proteases, which are released upon degranulation and are associated with NETs (29). Through the use of a mAb to histone–DNA complexes (PL2-3, H2A-H2B-DNA) (22), a significant reduction in intracellular H2A-H2B-DNA staining in vitro in transmigrated neutrophils was...
observed compared with in naive controls (Fig. 3ai–iii), implying that the decondensed DNA was released upon or after transmigration. Using PCR and specific mitochondrial or nuclear gene primers, we found that extracellular DNA was present in the conditioned medium of transmigrated neutrophils and the conditioned medium of naive neutrophils (Fig. 3bi, 3bii). The presence of extracellular decondensed DNA was confirmed by ELISA using the PL2-3 Ab: a significant increase in the abundance of decondensed DNA was detected in the conditioned medium of transmigrated neutrophils in comparison with the naive neutrophil-conditioned medium (Fig. 3c).

Neutrophil proteases contribute to tissue injury during inflammation (30–32) and brain injury after MCAo (21, 33, 34). Extensive work also showed that neutrophil elastase is associated with NETs (16, 35, 36). The mAb PL2-3 was also effective in identifying NETosis and associated inflammation in the periphery (16). After the direct transmigration of neutrophils onto neuronal cultures in the presence of PL2-3, an improvement in neuronal viability was observed using immunofluorescence: MAP2 staining was retained in neurons treated with the PL2-3 Ab: a significant increase in the abundance of decondensed DNA was detected in the conditioned medium of transmigrated neutrophils in comparison with the naive neutrophil-conditioned medium (Fig. 3c).

Neutrophil proteases mediate rapid neurotoxicity of transmigrated neutrophils

To evaluate the potential role of the released proteases and other substances in the neurotoxic mechanisms induced by transmigrated neutrophils, we first performed interventions using conditioned medium from transmigrated neutrophils. Pretreatment of neurons with MK-801 (NMDA receptor antagonist) partially, but significantly, reduced the neurotoxicity induced by conditioned medium from transmigrated neutrophils (Fig. 4a). Heat inactivation or trypsin treatment of conditioned medium from transmigrated neutrophils also inhibited its neurotoxic effect (Fig. 4a), suggesting a proteaceous neurotoxin.

Neutrophil-derived proteases are associated with NETs (36); therefore, we tested whether MMP-9, cathepsin-G, proteinase-3, or elastase, released from transmigrated neutrophils, may be responsible for the observed neurotoxicity. No effect was seen when specific inhibitors against certain proteases were used alone (data not shown). Because these proteases exhibit their actions in concert (37), a mixture of inhibitors against cathepsin-G (C), neutrophil elastase (E), proteinase-3 (aprotinin; A) and MMP-9 (SB3CT; M) was also tested (defined as CEAM, a mixture of inhibitors against cathepsin-G, neutrophil elastase, proteinase-3 [aprotinin], and MMP-9 [SB3CT]). CEAM did not affect neutrophil transmigration across IL-1-activated brain endothelium (Fig. 4b). Immunofluorescence showed maintained integrity, reduced degranulation, and less elastase/MMP-9 staining in transmigrated neutrophils in the presence of CEAM compared with vehicle-treated transmigrated neutrophils (Fig. 4cii, 4ciii). Neutrophils were directly migrated onto neuronal cultures across activated bEnd.5 cells in the presence of vehicle or CEAM for 2 h (Fig. 4dii, 4div). Importantly, neuronal structural integrity appeared to be maintained after the migration of transmigrated neutrophils in the presence of CEAM (Fig. 4div) in comparison with the vehicle-treated–transmigrated neutrophils (Fig. 4diii), thus supporting the link between neuronal toxicity and the proteases released from transmigrated neutrophils.

When transmigrated neutrophils were added directly to neurons, the presence of CEAM significantly rescued neuronal viability compared with the vehicle-treated–transmigrated neutrophil control (Fig. 4e). The presence of CEAM was ineffective when using conditioned medium from transmigrated neutrophils, suggesting that the cascade of events subsequent to full degranulation involving these neutrophil-derived proteases might result in neurotoxicity. This is supported by the observation that neurotoxicity could be successfully inhibited on site in neuronal cultures while maintaining transmigrated neutrophil integrity, but it could not be inhibited in conditioned medium from transmigrated neutrophils, in which it is possible that proteolytic events had already occurred, and full degranulation had taken place.

Dnase treatment of conditioned medium from transmigrated neutrophils failed to restore neuronal viability, indicating that DNA released from transmigrated neutrophils was not directly responsible for the observed neurotoxicity (Supplemental Fig. 2i). Several other inhibitors (calcium channel blocker nifidipene, broad-spectrum caspase inhibitor BOC-Asp(Ome)-FMK, cysteine protease inhibitor E64, protease-activated receptor inhibitor-1, L-α-aminoadipate, EDTA, EGTA, BAPTA to chelate-free calcium, apocynin to inhibit NAPDH oxidase, MEK1 inhibitor, the MAC-1 inhibitor clusterin, and the four individual CEAM components) all failed to rescue the
neuronal viability in the presence of conditioned medium from transmigrated neutrophils (data not shown).

These data indicate that neuronal viability can be maintained if neutrophil degranulation and release of multiple proteases are prevented in the presence of neurons. Time-lapse imaging indicated the presence of transmigrated neutrophils that were moving continually, as well as degranulated neutrophils that were in close association with neurons (Supplemental Video 2). Scanning electron microscopy was used to visualize the transmigrated neutrophils and neurons in greater detail (Fig. 4f–h, 4j). Transmigrated neutrophils were associated with the neurons (Fig. 4f) and were attached to intact neuronal cell bodies (Fig. 4gi, 4gii). In contrast, neuronal death, as evidenced by the presence of disintegrated neuronal cell bodies, was seen in the vicinity of collapsed neutrophils (Fig. 4gi, 4giii). Degranulation and NET release from transmigrated neutrophils were observed: the strand-like globular structures of the decondensed DNA were found protruding from the disintegrating neutrophil cell membrane (Fig. 4h, 4j).

Neutrophils recruited to the brain in vivo are associated with extracellular proteases and a loss of intracellular decondensed DNA

Decondensed DNA associated with neutrophils and loss of intracellular histone–DNA complexes were observed in animal models of neuroinflammation tested in vivo (Fig. 5a), such as after intracerebral LPS administration or MCAo (Fig. 5ai), MCAo in the presence of peripherally administered IL-1β (Fig. 5aii), MCAo preceded by systemic infection (Fig. 5aiii), and AMPA/IL-1-induced neurotoxicity (Fig. 5aiv). Within these models of neuroinflammation, intracellular PL2-3 staining was reduced or uneven in close proximity to the nuclear DNA staining, as indicated by DAPI (Fig. 5b). In general, the presence of neutrophil-associated decondensed DNA was reduced in the brain (80% of intracerebral neutrophils were PL2-3−) (Fig. 5ci, 5cii) in comparison with meningeal neutrophils (20% were PL2-3−). This provides further evidence for an altered state of recruited neutrophils in vivo. There was no significant difference in neutrophilic intracellular PL2-3 staining between different neuro-
Quantification revealed extracellular neutrophil-associated DNA (PL2-3) in 4 ± 1% of recruited neutrophils after intracerebral LPS administration, whereas a trend toward more neutrophils with extracellular PL2-3 was observed after AMPA/IL-1β–induced neurotoxicity, MCAo in the presence of peripherally administered IL-1β, or after MCAo preceded by systemic infection (9 ± 5%, 11 ± 5%, and 15 ± 5%, respectively).

Extracellular neutrophil-associated PL2-3 staining was mostly...
Neutrophil recruitment in acute brain injury is associated with extracellular proteases and loss of intracellular decondensed DNA in vivo. (a) Loss of intracellular histone–DNA complexes (PL2-3 staining) in neutrophils identified in the cerebral cortex in vivo in different established models of neuroinflammation after induction of cerebral ischemia (i), after induction of cerebral ischemia in the presence of peripherally injected IL-1β (ii), after induction of cerebral ischemia following systemic infection with *Trichuris muris* (iii), and after stereotactic intrastriatal administration of IL-1 and AMPA (iv) in rat. Scale bars, 5 μm. (b) In a population of recruited neutrophils in the inflamed brain, PL2-3 staining is uneven, and decondensed DNA is observed in close proximity to nuclear DNA (DAPI, blue). Scale bar, 10 μm. (c) Intracellular elastase associated with recruited neutrophils was observed in vivo, along the blood vessel wall and occasionally in the parenchyma after MCAo (Supplemental Fig. 4b). Disintegration of the perivascular basement membrane from the endothelial monolayer after MCAo was also observed in vivo (Supplemental Fig. 4c), indicating that products derived from recruited neutrophils could reach nearby neurons via the compromised BBB. In addition to this, populations of elastase+ neutrophils were observed perivascularly inside and outside the endothelial monolayer (lectin, blue) in the brain. Inset shows enlargement of area in box. Scale bar, 20 μm.

Discussion

We show in this study that transmigration through activated cerebrovascular endothelium critically alters neutrophils, leading to a proinflammatory, neurotoxic phenotype. Using established in vitro and in vivo models of neuroinflammation, we show the presence of released decondensed DNA (NETs) associated with key proteases after neutrophil transendothelial migration. Neurotoxicity of neutrophils was demonstrated previously, but over a 1–3 d period (13, 15), whereas, in this study, the transmigration-induced neurotoxicity developed very rapidly (within 30 min). Therefore, these data identify a novel neuroinflammatory mechanism: the development of rapid neurotoxicity of neutrophils by IL-1–induced cerebrovascular transmigration.

Neutrophil recruitment through endothelial activation is a common observation in inflammatory disease: the two-stage process of neutrophil activation of priming and mobilization leads to their adherence, rolling, and transmigration (6). The stimulation of this priming and recruitment can occur via the presence of pathogen associated molecular patterns or, in the case of sterile injury, damage associated molecular patterns produced by necrotic and apoptotic cells (5). Previous studies highlighting the importance of neutrophil recruitment in peripheral inflammation demonstrated a clear role for NETs through pathogen associated molecular patterns and damage associated molecular pattern stimulation linking both in vitro and in vivo observations (30–32).

Systemic inflammatory changes in response to cerebral ischemia also lead to the mobilization of peripheral inflammatory cells, which takes place in parallel to activation of the cerebrovascul ar endothelium (1, 17, 21). The release of neutrophils from the bone marrow in response to MCAo is associated with an increase in pro-MMP9 in circulating blood cells within hours (9). Activated neutrophils are recruited and adhere to inflamed blood vessels in the brain in response to neuroinflammation (38, 39). Their extravasation, which is associated with vascular leakage in the brain, was demonstrated with in vivo two-photon imaging (40). Intrastriatal injection of LPS or coinjection of AMPA and IL-1 into the striatum provided us additional in vivo paradigms of neuroinflammation that induce neutrophil recruitment and that are relevant to pathogen- and sterile-induced brain inflammatory conditions. Our in vivo data showed that most recruited neutrophils were associated with blood vessels in the ipsilateral hemisphere after MCAo and in excitotoxic- or endotoxin-induced neuroinflammation. Transmigrated neutrophils contained high levels of proinflammatory cytokines (e.g., KC and RANTES), indicating that such cells may contribute further to cerebrovascular activation and leukocyte recruitment to the brain.

The degranulation of neutrophils and the release of NETs containing decondensed DNA and proteases takes place normally as part of an antibacterial defense (16, 36, 41, 42). This might be initiated in vivo to prevent the bacterial invasion of seriously injured tissues. These properties could contribute to the poor out-
come observed in stroke patients with systemic inflammation (43, 44). Murine in vivo models also reflect this, because the upregulation observed in neutrophil recruitment and neutrophil-derived proteases might account for BBB damage and increased neuronal death (4, 21). Through the release of decondensed DNA and proteases during extravasation, these neurotoxic products could reach neurons through the disrupted basement membrane of the glia limiting and lead to their demise. Additional stimuli received from parenchymal cells or extracellular matrix may also exacerbate the effects of the transmigrated neutrophils. Although neutrophils are not recruited to the brain until several hours after acute brain injury, once transendothelial migration takes place, neutrophils can exert rapid toxicity (within 30 min, based on our in vitro data) to neurons. Therefore, it is not surprising that powerful mechanisms have evolved to prevent parenchymal infiltration of neutrophils, such as the complex barrier structures of the neurovascular unit or phagocytosis of invading neutrophils by microglia (45, 46).

Although our data suggest that the process of transmigration itself is a sufficient stimulus to endow neutrophils with neurotoxic properties and, thus, the ability to kill neurons immediately, it does not imply that diapedesis of neutrophils is the sole factor that contributes to their neurotoxicity. Adherence to activated brain endothelium, partial diapedesis, or the initiation of transmigration could also contribute to the development of a neurotoxic phenotype in vivo; these mechanisms warrant further investigation. Proinflammatory alterations in neutrophils induced by their transmigration may reflect a heightened state of activation, such that they are primed to respond to additional stimuli, which may indirectly contribute to further neurotoxicity in vivo. Similarly, the pro-survival effect of transmigration on neutrophils is likely to further exacerbate the neurotoxic potential of neutrophils in the injured brain. Although the release of NETs often occurs in association with neutrophil death, recent data indicate that viable neutrophils can form NETs, which parallels the release of mitochondrial, but not nuclear, DNA (35). This is strikingly similar to our findings: release of proteases and mitochondrial DNA in neutrophil-conditioned medium and in vivo and the restricted release of nuclear DNA released in the brain. This might indicate that the release of proteases and decondensed DNA from neutrophils in the brain is an active process and not simply a consequence of neutrophil death. It is not known when the release of proteases and DNA takes place in vivo, but most recruited neutrophils lacked intracellular PL2-3 immunopositivity in the brain. It is also possible that classical NETosis involving the release of nuclear DNA, as observed in vitro or in peripheral tissues, is restricted in the brain or such cells are rapidly phagocytosed by resident microglia.

Our data also show that the neurotoxic phenotype of neutrophils after transendothelial migration is not unique to activated brain endothelium, because conditioned medium of neutrophils collected in vivo after thioglycollate-induced peritonitis also exerts toxicity in neuronal cultures. Irrespective of whether neurotoxicity is triggered by peripheral or central transendothelial migration, neutrophils recruited to the brain parenchyma can exert neurotoxicity, which is likely to be further increased by the fact that toxic products could reach nearby neurons from transmigrating neutrophils if the BBB is compromised. Our in vitro data show that neuron–neutrophil contact is not required for neutrophil neurotoxicity. Furthermore, peripherally located transmigrating neutrophils could exert toxicity to neurons outside the brain as well, which has to be further investigated in the context of peripheral neuropathies.

Elastase, cathepsin-G, and proteinase-3 are known to be present in neutrophil primary granules, whereas tertiary granules contain, among other components, MMP-9 (47). Other studies showed the localization of NETs with elastase, cathepsin-G, and proteinase-3 (16, 42, 48), which suggests that the release of primary granules and NETs is the last line of defense for a neutrophil before apoptosis (29). The formation of NETs provides a link between the released proteases and decondensed DNA: the presence of elastase was identified attached to the NET itself (16, 49). Elastase is involved in the very first stages of nuclear DNA decondensation through its exit from the primary granules directly to the nucleus (48). The fact that CEAM was not protective when added to conditioned medium of transmigrated neutrophils indicates that a cascade of events, subsequent to protease and decondensed DNA release, is responsible for neurotoxicity; this needs to be investigated in further studies. Similarly, considerable further work is required to establish the functional link between mitochondrial DNA and protease activation in neutrophils during NETosis, although it is relevant that mitochondrial DNA can activate human neutrophils, resulting in the release of proteases, such as MMP-9 (50).

Our in vitro imaging data and scanning electron microscopic observations indicated that transmigrated neutrophils are closely associated with neurons. This accords with the fact that inhibition of the proteases present after degranulation is successful only if transmigrated neutrophils are applied directly to neuronal cultures and not when in the presence of just the conditioned medium of transmigrated neutrophils. This implies that proteolytically cleaved products of these proteases are neurotoxic. CEAM provided almost full protection against neurotoxicity when applied in the presence of transmigrated neutrophils, indicating that proteases are the main mediators of toxicity in this model. The possibility that the actual presence of neurons triggers the degranulation of neutrophils is not known and remains to be determined in future studies.

In conclusion, we show that neutrophils that have migrated across an activated cerebrovascular endothelium profoundly change their phenotype and secrete decondensed DNA and proteases that contribute to neuronal death. IL-1 has a key role in cerebrovascular activation, neutrophil recruitment, and transendothelial migration, which contribute to brain inflammation and neuronal death. Therefore, inhibition of neutrophil recruitment and extravasation from cerebral vessels by blocking IL-1 actions might be a preferable strategy to inhibiting the various factors released by neutrophils once they have migrated, because it is likely to offer more effective protection against neutrophil-mediated neuronal death, a significant component in ischemic and other inflammatory brain injuries. Therefore, these findings further support the development of inhibitors of IL-1 as neuroprotective agents.

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