Ultra-Sensitive Molecular MRI of Vascular Cell Adhesion Molecule-1 Reveals a Dynamic Inflammatory Penumbra After Strokes

Maxime Gauberti, PhD*; Axel Montagne, PhD*; Oscar A. Marcos-Contreras, PhD; Audrey Le Béhot, MSc; Eric Maubert, PhD; Denis Vivien, PhD

Background and Purpose—Our aim was to assess the spatiotemporal evolution of the cerebrovascular inflammation occurring after ischemic and hemorrhagic strokes using a recently developed, fast, and ultra-sensitive molecular MRI method.

Methods—We first assessed longitudinally the cerebrovascular inflammation triggered by collagenase-induced hemorrhage and by permanent/transient middle cerebral artery occlusion in mice, using MRI after injection of microparticles of iron oxide targeted to vascular cell adhesion molecule-1 (MPIOs-αVCAM-1). Thereafter, we used this method to study the anti-inflammatory effects of celecoxib, atorvastatin, and dipyridamole after stroke.

Results—Using multiparametric MRI, we demonstrated that the level and the kinetics of cerebrovascular VCAM-1 expression depend on several parameters, including stroke pathogenesis, the natural history of the disease, and the administration of inflammation-modulating drugs. Interestingly, in transient middle cerebral artery occlusion and intracranial hemorrhage models, VCAM-1 expression was maximal at 24 hours and almost returned to baseline 5 days after stroke onset. In contrast, after permanent middle cerebral artery occlusion, VCAM-1 overexpression was sustained between 24 hours and 5 days, and was particularly significant in the peri-infarct areas. Our results suggest that these perilesional areas expressing VCAM-1 constitute an inflammatory penumbra that is recruited by the ischemic core during the subacute phase. Using MPIOs-αVCAM-1–enhanced imaging, we also provided evidence that celecoxib and atorvastatin (but not dipyridamole) alleviate VCAM-1 overexpression after stroke and prevent formation of the inflammatory penumbra.

Conclusions—MPIOs-αVCAM-1–enhanced imaging seems to be promising in the detection of individuals presenting with severe cerebrovascular responses after stroke, which could therefore benefit from anti-inflammatory treatments. (Stroke. 2013;44:1988-1996.)

Key Words: MRI • neuroinflammation • stroke • VCAM

Inflammation is considered to be an integral part of the pathogenic processes occurring after stroke, being involved in the early deleterious cascades and also in reorganization and repair of the poststroke brain. Despite promising results in experimental studies, all inflammatory modulating treatments have failed in stroke clinical trials. To explain this apparent paradox, one may consider the variability of poststroke inflammatory responses among individuals. Indeed, the severity of the inflammatory response may depend on different parameters, including prestroke status of the patient and the natural history of the disease. Accordingly, experimental studies revealed that animals with systemic disorders (such as obesity or sepsis) present a primed inflammatory state, which exacerbates the inflammatory response after stroke. Moreover, persisting arterial occlusion seems to exacerbate the expression of inflammatory markers in the poststroke brain. Because immunomodulation could be harmful in some patients by increasing the risk of infection (such as pneumonia), the use of anti-inflammatory treatment should be restricted to those presenting severe and sustained inflammatory responses to maximize the benefit/risk ratio.

To this aim, several imaging approaches have been developed to monitor neuroinflammatory processes. For example, in situ detection of inflammation-related proteins by molecular imaging has been successful in several neurological pathologies, using positron emission tomography. However, the online-only Data Supplement is available with this article at http://stroke.ahajournals.org/lookup/suppl/doi:10.1161/STROKEAHA.111.000544/-/DC1. 

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From the Institut National de la Santé Et de la Recherche Médicale (INSERM) U919 Serine Protease and Pathophysiology of the Neurovascular Unit, University Caen Basse-Normandie, GIP Cyceron, Bd Becquerel, BP5229, Caen, France.
*Drs Gauberti and Montagne contributed equally to this work.
Correspondence to Denis Vivien, PhD, INSERM U919 Serine Protease and Pathophysiology of the Neurovascular Unit, University Caen Basse-Normandie, GIP Cyceron, Bd Becquerel, BP5229, Caen, France. E-mail vivien@cyceron.fr
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limited availability and low spatial resolution impair the use of positron emission tomography in routine clinical practice. To overcome these limitations, molecular MRI of inflammation using ultrasmall particles of iron oxide has been described in preclinical and clinical studies. Nevertheless, this approach has significant limits, which preclude its use in the acute and subacute phases of stroke, including an uncompressible time between injection and imaging (>24 hours to allow plasmatic clearance of the iron particles), a passive extravasation through altered blood–brain barriers (which could lead to false-positive findings in both acute and subacute phases), and a low sensitivity to detect the early steps of inflammation.

We recently developed an original contrast agent targeting the vascular cell adhesion molecule-1 (VCAM-1), which displays all the required characteristics of an ideal imaging agent, thus allowing a rapid and sensitive assessment of the early stages of the inflammatory reaction. In the present study, we investigated the efficiency of these microsized particles of iron oxide targeting VCAM-1 (MPIOs-αVCAM-1) to monitor cerebrovascular inflammation and evaluate the response to anti-inflammatory treatments after ischemic and hemorrhagic strokes.

Materials and Methods
The Materials and Methods section is available as an online supplement.

Results
Inflammatory Response Differs Between Permanent and Transient Middle Cerebral Artery Occlusion as Assessed by MPIOs-αVCAM-1 Imaging
Using MPIOs-αVCAM-1–enhanced imaging, we first aimed to compare temporal and spatial evolutions of cerebrovascular inflammation after permanent middle cerebral artery occlusion (pMCAo; by electrocoagulation) and transient MCAo (tMCAo; by microinjection of thrombin leading to intra-arterial thrombus formation followed by a spontaneous reperfusion; Figure I in the online-only Data Supplement). These 2 models mimic 2 different natural histories of acute ischemic stroke: patients may undergo spontaneous reperfusion of the thrombosed artery because of endogenous thrombolytic mechanisms (tMCAo), or the artery may remain occluded (pMCAo). In the pMCAo model, MPIOs-αVCAM-1–enhanced imaging revealed a sustained VCAM-1 overexpression in the entire brain from +24 hours to +5 days after ischemic onset (Figure 1A and Figures II and III in the online-only Data Supplement), with a cerebrovascular inflammation especially important in the peri-infarct area (yellow arrows in Figure 1A). Longitudinal studies in the pMCAo model demonstrated that VCAM-1 expression did not change significantly between +24 hours and +5 days in the ipsilateral cortex (from 42.6% at +24 hours to 41.0% at +5 days) but slightly decreased in the contralateral hemisphere (from 13.7% at +24 hours to 9.6% at +5 days; P<0.05). In contrast, in the tMCAo model, VCAM-1 expression was almost exclusively found in the ischemic lesion and gradually decreased over time (signal void volume decreased from 31.2% at +24 hours to 8.4% of the ipsilateral cortex at +5 days; P<0.05; Figure 1A through 1D). These results were replicated in 2 other models of permanent and tMCAo (Figure IV in the online-only Data Supplement). Overall, these results demonstrate that the cerebrovascular inflammation after pMCAo is strong and sustained, whereas the cerebrovascular inflammation after thrombin-induced tMCAo is mild, restricted to the ischemic lesion, and normalizes rapidly.

MPIOs-αVCAM-1 Predict Delayed Postischemic Injury After MCAo
Thereafter, we investigated whether the inflammatory areas revealed by MPIOs-αVCAM-1–enhanced imaging represent at-risk tissues, which are susceptible to be recruited by the lesion core in the subacute phase. To this aim, we performed MPIOs-αVCAM-1–enhanced imaging at +24 hours and followed the evolution of the stroke-induced lesion by longitudinal T2-weighted imaging. As previously described, the ischemic lesion size significantly increased in the subacute stage after pMCAo (+22.7% between +24 hours and +72 hours; P<0.05; Figure 2A). According to the results in Figure 1, at +24 hours, MPIOs-αVCAM-1–enhanced imaging revealed an especially strong VCAM-1 overexpression in spatially restricted areas located around the initial ischemic lesion (yellow arrows on Figure 2A). Interestingly, a significant part of these highly VCAM-1–expressing areas were subsequently recruited by the ischemic core between +24 hours and +72 hours after pMCAo (Figure 2B and 2C). Consistently, similar findings were obtained in another model of permanent ischemia (Figure IV in the online-only Data Supplement). Immunohistological studies performed +24 hours after pMCAo confirmed the presence of numerous VCAM-1–positive vessels with bound MPIOs-αVCAM-1 at the periphery of the ischemic lesion in MAP-2–positive areas (Figure 2D). Moreover, at +72 hours after pMCAo, numerous activated microglial cells were located in the periphery of the lesion, suggesting that parenchymal inflammatory processes occur in this area (Figure V in the online-only Data Supplement). Altogether, these results suggest that the perilesional parenchyma expressing VCAM-1 represent an inflammatory penumbra, which is susceptible to recruitment by the ischemic core in a delayed fashion. The mean fraction of the inflammatory penumbra, which was recruited by the lesion core between +24 hours and +72 hours, was 71% in the electrocoagulation model. However, we cannot exclude a more delayed recruitment of the remaining areas because the extensive lesion remodeling occurring at +5 days precludes accurate localization of the inflammatory penumbra. Importantly, in areas devoid of VCAM-1 overexpression (green dotted line in Figure 2C), no lesion growth was detected.

Ischemia/Oligemia Rather Than Neuronal Cell Death May be the Main Trigger of VCAM-1 Upregulation After Stroke
Current knowledge indicates that early cerebrovascular inflammation after brain damage is triggered by 2 different mechanisms: release of damage-associated molecular patterns and potassium by dying cells, and direct impact of ischemia/oligemia on endothelial cells,
triggering upregulation of cell adhesion molecules. To further investigate the respective contributions of damage-associated molecular patterns and ischemia/oligemia on cerebrovascular inflammation, we compared VCAM-1 upregulation in 2 experimental paradigms: in the first one, we induced oligemia without brain damage (common carotid artery ligation model). In the second, we induced brain damage without ischemia/oligemia (N-Metyl-D-Aspartate injection model). Interestingly, VCAM-1 upregulation 24 hours after surgery was more important in the common carotid artery ligation model (Figure VI in the online-only Data Supplement). This result suggests that after ischemic stroke, which involves both ischemia/oligemia and brain damage, cell-death–independent effects of ischemia/oligemia are mainly responsible for VCAM-1 upregulation. Therefore, the differential pattern of VCAM-1 upregulation in transient and permanent ischemia may be explained by a longer and more severe period of ischemia.

MPIOs-αVCAM-1 Reveal the Anti-Inflammatory Effects of Atorvastatin After Ischemic Stroke

Thereafter, we investigated whether MPIOs-αVCAM-1 could be used to assess the effects of anti-inflammatory drugs on poststroke VCAM-1 expression. We chose celecoxib, atorvastatin, and dipyridamole as 3 potential anti-inflammatory treatments because previous studies had demonstrated their vasoprotective effects.12–14 Thus, mice received either 5 mg/kg of celecoxib (intraperitoneal or an equivalent volume of 50% dimethyl sulfoxide in saline), 80 mg/kg of atorvastatin per os (or an equivalent volume of water), 100 mg/kg of dipyridamole (intraperitoneal or an equivalent volume of 5% dimethyl sulfoxide in saline) at +6 hours, +24 hours, and +48 hours after pMCAo. As hypothesized, MPIOs-αVCAM-1–enhanced imaging revealed a lower level of VCAM-1 expression in celecoxib and atorvastatin-treated mice than in control mice +24 hours after pMCAo (Figure 3A through 3D). This reduction was evident in the contralateral cortex but did not reach statistical significance in the ischemic cortex. Interestingly, although the ischemic lesion size at +24 hours did not differ significantly (data not shown), the celecoxib- and atorvastatin-treated mice displayed smaller lesion growth between +24 hours and +72 hours than the corresponding control mice, supporting a role for cerebrovascular inflammation in the delayed expansion of the lesion (Figure 3B). Additional immunohistological studies were performed at +24 hours in atorvastatin-treated mice and

Figure 1. Inflammatory response differs between permanent and transient middle cerebral artery occlusion (MCAo), as assessed by microparticles of iron oxide targeted to vascular cell adhesion molecule-1 (MPIOs-αVCAM-1) imaging. A, Representative images of longitudinal molecular imaging of VCAM-1 revealing diffuse brain inflammation after permanent MCAo (pMCAo). VCAM-1 expression gradually decreased in the contralateral side but persisted at least 5 days in the ischemic cortex. B, Corresponding quantification of the MPIOs-αVCAM-1–induced signal void in the entire ipsilateral or contralateral cortices at +24 h, +72 h, and +5d after pMCAo (n=6; C). D, The same as in A and B, but for transient MCAo (tMCAo) induced by intra-arterial injection of thrombin (n=6). The orange dotted lines represent the ischemic lesion as visualized on the T2-weighted images for each imaging time point. Because the same animals were longitudinally imaged, statistical analyses were performed using the Wilcoxon signed-rank test. ns indicates not significant.
confirmed the MRI results by revealing a smaller proportion of VCAM-1–positive vessels in atorvastatin-treated than in control mice in the nonischemic hemisphere and in the periphery of the lesion but not in the ischemic core (Figure 4A through 4C). High-magnification immunohistological studies confirmed the presence of bound MPIOs-αVCAM-1 on the VCAM-1–expressing vessels of the ischemic lesion at +24 hours (Figure 4D). In contrast to celecoxib and atorvastatin, dipyridamole treatment did not influence MPIOs-αVCAM-1–induced signal voids, suggesting a lack of significant anti-inflammatory effects in these experimental settings (Figure VII in the online-only Data Supplement), as previously demonstrated. However, early tissue-type plasminogen activator administration did not significantly influence poststroke cerebrovascular inflammation as assessed by MPIOs-αVCAM-1 enhanced imaging. The effect of late thrombolysis on cerebrovascular inflammation deserves further investigation.

**MPIOs-αVCAM-1 Reveal Peri-Hematoma Inflammation in a Model of Intracranial Hemorrhages**

In the second part of this study, we investigated whether MPIOs-αVCAM-1–enhanced imaging could detect cerebrovascular inflammation after hemorrhagic stroke. To this aim, we performed longitudinal MPIOs-αVCAM-1–enhanced imaging of mice at +24 hours, +72 hours, and +5 days after collagenase-induced intracranial hemorrhages or intrastriatal saline injection (Figure 5A). After MPIOs-αVCAM-1 injection, T2*-weighted imaging revealed numerous signal voids corresponding to VCAM-1–expressing brain vessels in both ipsilateral and contralateral hemispheres at +24 hours (Figure 5B). There were significantly more MPIOs-αVCAM-1–induced signal voids in the nonischemic hemisphere and in the periphery of the lesion but not in the ischemic core (Figure 5C). High-magnification immunohistological studies confirmed the presence of bound MPIOs-αVCAM-1 on the VCAM-1–expressing vessels of the ischemic lesion at +24 hours (Figure 5D). In contrast to celecoxib and atorvastatin, dipyridamole treatment did not influence MPIOs-αVCAM-1–induced signal voids, suggesting a lack of significant anti-inflammatory effects in these experimental settings (Figure VII in the online-only Data Supplement).
voids in collagenase-treated than in saline-treated mice at +24 hours and at +72 hours (Figure 5B and Figure IX in the online-only Data Supplement). Five days after hemorrhage induction, the volume of MPIOs-αVCAM-1-induced signal voids almost returned to baseline, but was still significantly higher than in saline-treated mice (Figure 5C). In mice injected with control MPIOs-αIgG, no signal void was present, apart from the hematoma itself (Figure 5B). On immunohistological examination at +72 hours, numerous Iba-1–positive cells (microglia/macrophages) were detected inside the hematoma (especially at the inner periphery; orange arrows on Figure 5D), whereas glial fibrillary acidic protein-positive cells (reactive astrocytes, yellow arrowheads) were concentrated at the outer periphery of the hemorrhage. VCAM-1 immunostaining confirmed the MRI findings by revealing numerous VCAM-1 expressing vessels outside the hematoma (Figure 5D). Altogether, these data demonstrate the efficiency of molecular MRI of VCAM-1 to evaluate cerebrovascular inflammation after hemorrhagic stroke.

MPIOs-αVCAM-1 Reveal the Anti-Inflammatory Effects of Atorvastatin After Hemorrhagic Stroke

Subsequently, we investigated whether MPIOs-αVCAM-1 could reveal the anti-inflammatory effects of acute atorvastatin treatment on cerebrovascular VCAM-1 expression after intracranial hemorrhage. To this aim, we administered 80 mg/kg of atorvastatin PO at +6 hours, +24 hours, and +48 hours after induction of intracranial hemorrhage. Thereafter, we performed molecular imaging of VCAM-1 expression statistically significant at +72 hours (Figure 6A through 6C). Whereas the mean signal void volume was 14.3% and 13.5% of the brain in
control mice at +24 hours and +72 hours, respectively, it was 10.9% and 5.15% in atorvastatin-treated mice (Figure 6C). These results confirm that atorvastatin treatment displays anti-inflammatory effects after hemorrhagic stroke in mice. Moreover, they demonstrate the efficiency of MPIOs-αVCAM-1 in evaluation of the efficacy of anti-inflammatory treatments after intracranial hemorrhage.

Discussion

In the present study, we report for the first time, to our knowledge, a molecular MRI method allowing rapid, sensitive, reliable, and longitudinal assessment of cerebrovascular inflammation after stroke. We demonstrated that MPIOs-αVCAM-1–enhanced imaging provides unique information about the severity of the cerebrovascular inflammatory response occurring after stroke. Our results suggest that this information may help to improve our current management of stroke patients.

Using MPIOs-αVCAM-1–enhanced imaging, we showed that VCAM-1 expression after cerebrovascular events varies spatially and temporally, depending on the ischemic stroke subtype. Whereas in transient ischemic stroke, cerebrovascular inflammation almost completely regressed 5 days after stroke onset, VCAM-1 overexpression was sustained and particularly significant in the peri-infarct area in the permanent model. These results are supported by a recent study demonstrating sustained inflammatory markers after permanent ischemia.6 We also show that VCAM-1–positive peri-infarct areas were recruited by the ischemic core in the subacute phase, suggesting the existence of an inflammatory penumbra. Although we did not demonstrate a causal relationship between overexpression of VCAM-1 in the peri-infarcted area and the extension of the ischemic lesion, this relationship is supported by a previous study by Liesz et al,11 who demonstrated that the delayed lesion growth after pMCAo is because of a VCAM-1–dependent blood–brain barrier diapedesis of T-lymphocytes, which exert neurotoxic effects once in the brain parenchyma. More recently, Huang et al also demonstrated that an anti-inflammatory drug (C-X-C chemokine receptor type 4 antagonist) inhibited delayed lesion growth (24 to 72 hours) in another permanent ischemia model.17 Interestingly, the particular kinetics of this delayed infarction suggests that patients presenting an inflammatory penumbra at 24 hours may still benefit from therapy targeting the interaction between leukocytes and the vascular wall (such as the multiple-sclerosis drug Natalizumab).11

In the subacute stage of ischemic stroke, we demonstrated that celecoxib and atorvastatin alleviate cerebrovascular inflammation and prevent formation of the inflammatory penumbra. Because statins are safely administered as
prophylactic drugs in poststroke patients (at much smaller
doses), our results support the fact that ischemic stroke
patients could benefit from statin administration in the early
phase to limit potential delayed inflammatory damages.18
It is interesting to note that a recent meta-analysis of statin
efficiency in experimental models of ischemic stroke indicates
that statin therapy may be more efficient in permanent than
in transient models.19 Our results using MPIOs-αVCAM-1–
enhanced imaging suggest that this discrepancy could
be explained by the more sustained inflammatory response
occurring after pMCAo than after tMCAo. Because the effect
of statins is pleiotropic, whether the observed preventive
effects on delayed lesion growth are solely caused by the
downregulation of VCAM-1 deserves further investigation.
Altogether, these data emphasize the interest of molecular
imaging to select subgroups of patients who may benefit from
anti-inflammatory therapy, thereby improving the benefit/risk
profile of the treatment.

We also demonstrated that MPIOs-αVCAM-1 allow non-
invasive assessment of cerebrovascular inflammation after
collagenase-induced intracranial hemorrhage. Because high
concentration of soluble VCAM-1 in the cerebrospinal fluid
is associated with poor outcome in patients with hemor-
rhagic stroke,20 it has been suggested that particular subsets of
patients presenting strong overexpression of VCAM-1 could
benefit from anti-inflammatory treatment. MPIOs-αVCAM-1–
enhanced imaging could be useful in identifying these patients
to test innovative anti-inflammatory strategies. One such
strategy could be the administration of statins, which showed
potential therapeutic efficiency in patients with subarach-
noid hemorrhages.21 Indeed, we demonstrated here that statin
administration significantly alleviates the cerebrovascular
inflammation after intracranial hemorrhage. These results confirm previous experimental studies and support further development of this therapeutic strategy for clinical use.22

A potential limit of MPIOs-based imaging (common to all negative contrast agents) is the occurrence of false-positive findings because of disease-related T2* effects (such as hemorrhages; Figure 5), which should be carefully identified on precontrast images. Moreover, the currently used MPIOs are not biodegradable and cannot be used in clinical studies. Therefore, clinical translation of this method would require production of biocompatible MPIOs. Whether MPIOs-αVCAM-1 could interfere with the binding of leukocytes to the vascular wall also deserves further investigations.

In conclusion, we demonstrate here that MPIOs-αVCAM-1-enhanced imaging allows evaluation of the poststroke cerebrovascular inflammation in a timely and highly sensitive manner. Notably, the cerebrovascular inflammation is particularly severe in the intact areas surrounding the ischemic lesion after pMCAo, suggesting the presence of an inflammatory penumbra that is recruited by the lesion core in the subacute phase. Altogether, our results suggest that the presence of an inflammatory penumbra after ischemic stroke could constitute an imaging criterion to select patients for subacute anti-inflammatory treatments.

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**Disclosures**

None.

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**References**

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SUPPLEMENTAL MATERIAL

Ultra-sensitive molecular MRI of VCAM-1 reveals a dynamic inflammatory penumbra following stroke.

Maxime Gauberti*, Axel Montagne*, Oscar Marcos-Contreras, Audrey Le Béhot, Eric Maubert and Denis Vivien

Expanded Materials and Methods

Supplementary Figure 1: Spontaneous and tPA-induced recanalisation in the thrombin model.

Supplementary Figure 2: Binding half-life of MPIOs-αVCAM-1 in a permanent stroke model (FeCl₃).

Supplementary Figure 3: Comparison of MPIO-IgG and MPIO-αVCAM-1 induced signal voids in the pMCAO model.

Supplementary Figure 4: Inflammatory response differs between permanent and transient MCAo as assessed by MPIOs-αVCAM-1 imaging.

Supplementary Figure 5: Numerous activated microglial cells are present in the recruited inflammatory penumbra 72 hours after MCAo.

Supplementary Figure 6: Oligemia induces stronger upregulation of VCAM-1 than excitotoxic neuronal cell-death.

Supplementary Figure 7: Dipyridamole does not alleviate VCAM-1 expression following pMCAo.

Supplementary Figure 8: Influence of tPA-induced thrombolysis on cerebrovascular inflammation.

Supplementary Figure 9: Comparison of MPIO-αVCAM-1 induced signal voids following saline and collagenase injection in the striatum.
Expanded Material and Methods

Targeting-moiet ties conjugation to MPIO

Microparticles of iron oxide (MPIO; diameter 1.08 µm) with p-toluenesulphonyl reactive surface groups (Invitrogen) were covalently conjugated to purified monoclonal rat anti-mouse antibodies for VCAM-1 as previously described\(^1\). Characterization of the MPIO-αVCAM-1 has been performed previously\(^1\). Mice received intravenous injection of 1.0 mg/kg (equivalent Fe) of conjugated MPIOs for contrast-enhanced MRI. Imaging was performed 20 minutes after particles administration as described above.

Mice

Experiments were performed on male Swiss mice (for intracranial hemorrhage model) or on male C57Bl/6 mice (for permanent and transient MCAo) (38 ± 5 g and 29 ± 3 g respectively, produced and provided by our local animal facilities, CURB, France) in accordance with European communities Council (Directives of November 24, 1986 (86/609/EEC)) and French Legislation (act no. 87-848) on Animal Experimentation. We chose to use two different strains of mice to further validate the applicability of our imaging method. Mice were deeply anesthetized with isoflurane 5% and maintained under anesthesia with 2% isoflurane in a 70% / 30% gas mixture (N\(_2\)O/O\(_2\)) during surgery. The rectal temperature was maintained at 37 ± 0.5°C throughout the surgical procedure using a feedback-regulated heating system. All MRI and immunohistological analyses, including signal voids quantification (see below), were performed blinded to the experimental data.

Permanent and transient middle cerebral artery occlusion (MCAo)

C57BL6/J male mice were placed in a stereotaxic frame, the skin between the right ear and the right eye was incised, and the temporal muscle was carefully retracted. For permanent MCAo, the MCA was exposed and permanently occluded by electrocoagulation or FeCl\(_3\) (Sigma-Aldrich) was topically applied on the MCA (as previously described\(^2\)). For transient MCAo, 1 I.U. of murine recombinant thrombin (Kordia, Leiden) was injected directly into the exposed MCA to induce formation of a clot\(^3\). In this model, the thrombotically occluded MCA recanalizes spontaneously\(^4\) (see also Supplementary Figure 1). Alternatively, we induced mechanical pressure using a glass micropipette to occlude the MCA during 45 minutes. In this transient model, occlusion and reperfusion was monitored by laser Doppler Flowmetry.

Intracranial hemorrhage model

A unilateral striatal injection of collagenase type VII\(^5\) (0.045 U in 0.5 µL of saline; Sigma-Aldrich) was performed after placing Swiss male mice in a stereotaxic frame (coordinates: 0.5 mm anterior, 2.0 mm lateral, -3 mm ventral to the bregma). Solutions were injected by the use of a glass micropipette to minimize hemorrhage-unrelated tissular damage. MPIOs-αVCAM-1-induced signal void were quantified in the peri-hematoma area (ipsilateral cortex).

Dipyridamole treatment

Dipyridamole (100 mg/kg, Sigma-Aldrich) or saline with 5% dimethyl-sulfoxide (Sigma-Aldrich) was injected intraperitoneally at +6h, +24h and +48h post pMCAo. Randomization was performed after surgery (collagenase-induced hemorrhage) and no animal was excluded.

Celecoxib treatment
Celecoxib (Sigma-Aldrich) was dissolved in DMSO and administered i.p. (5 mg/kg) after further dilution in saline (50%/50%). Control animals received the vehicle alone. Treatment was given at +3h, +24h and +48h post stroke. Randomization was performed after surgery (pMCAo by electrocoagulation) and no animal was excluded.

Atorvastatin treatment

Atorvastatin (Tahor, Pfizer) was dissolved in distilled water and administered per os (80 mg/kg). In both hemorrhagic and ischemic models, atorvastatin or distilled water (control) was given at +6h, +24h and +48h post stroke. Randomization was performed after surgery (pMCAo or collagenase-induced hemorrhage) and no animal was excluded.

Magnetic resonance imaging

Experiments were carried out on a Pharmascan 7 T/12 cm system using surface coils (Bruker, Germany). T2-weighted images were acquired using a MSME sequence: TE/TR 51ms/2500ms with 70µm*70µm*500µm spatial resolution. 3D T2*-weighted gradient echo imaging with flow compensation (GEFC, spatial resolution of 93µm*70µm*70µm interpolated to an isotropic resolution of 70 µm) with TE/TR 12.6ms/200ms and a flip angle (FA) of 24° was performed to visualize MPIOs (acquisition time= 15 min). All T2*-weighted images presented in this study are minimum intensity projections of 4 consecutive slices (yielding a Z resolution of 280 µm). Lesion sizes were quantified blinded to the experimental data on T2-weighted images and signal voids quantification on 3D T2*-weighted images using automatic Otsu tresholding in ImageJ software (v1.45r). Results are presented as volume of MPIOs-induced signal void divided by the volume of the structure of interest (in percent). Importantly, there was no difference between T2-weighted and diffusion-weighted imaging in the experiments performed (data not shown). Moreover, imaging was well correlated with immunohistological findings in the different experimental models performed Magnetic resonance angiographies (MRA) were performed using a 2D-TOF sequence (TE/TR 10/50 ms). Analyses of the MCA MRA were performed blinded to the experimental data using the following score: 2: normal appearance, 1: partial occlusion and 0: complete occlusion of the MCA. When appropriate, mice randomly received MPIO-IgG or MPIO-αVCAM-1. The delay between two MPIO-αVCAM-1 administrations was superior to 24 hours in all the experiments to allow complete washout of the bound particles (Supplementary Figure 2).

Immunohistochemistry

Deeply anesthetized mice were transcardially perfused with cold heparinized saline (15 mL) followed by 150 mL of fixative (PBS 0.1 M, pH 7.4 containing 2% paraformaldehyde and 0.2% picric acid). Brains were post-fixed (18 hours; 4°C) and cryoprotected (sucrose 20% in veronal buffer; 24 hours; 4°C) before freezing in Tissue-Tek (Miles Scientific, Naperville, IL, USA). Cryomicrotome-cut transversal sections (8-10 µm) were collected on poly-lysine slides and stored at – 80°C before processing. Sections were co-incubated overnight with rat monoclonal anti-mouse VCAM-1 (1:1500; from AbD Serotec), goat anti-collagen-type IV (1:1500; Southern Biotech), chicken anti-MAP-2 (1:8000; Abcam), goat anti-Iba-1 (1:800; Abcam) or rabbit anti-GFAP (1:800; Dako) in veronal buffer (pH 7.4). Primary antibodies were revealed using Fab’2 fragments of Donkey anti-rat, goat, chicken or mouse IgG linked to FITC, TRITC or DyLight 629 (1:500, Jackson ImmunoResearch, West Grove, USA). Washed sections were coverslipped with antifade medium containing DAPI and images were digitally captured using a Leica DM6000 microscope-coupled coolsnap camera and visualized with Metavue 5.0 software (Molecular Devices, USA) and further processed using ImageJ 1.45r software (NIH).
Quantification of VCAM-1 positive vessels

The image analysis was performed using ImageJ software. The proportion of vessels expressing VCAM-1 was obtained by dividing the number of VCAM-1+ vessels on immunohistological images by the total number of vessels (as revealed by collagen type IV immunostaining) in three randomly selected slices per animal. The ischemic core was located using MAP-2 immunostaining (infarcted tissue does not express MAP-2) and the periphery of the lesion was defined as the area distant from less than 500 µm from the limit between ischemic and non-ischemic tissues. The images corresponding to the non-ischemic tissue was taken at the opposite of the ischemic lesion, in the contralateral cortex. A vessel was considered VCAM-1 positive when the fluorescence signal was distinguishable from the background (parenchymal auto-fluorescence). All analyses were performed blinded to the experimental data.

Statistical analyses

Results are the mean ± SD. Statistical analyses were performed using Kruskal-Wallis (for multiple comparisons) followed by Mann-Whitney’s U-test. For longitudinal experiments, statistical analyses were performed using the Wilcoxon signed-rank test. Statistical significance was concluded for p<0.05.
References


Supplementary Figure 1: Spontaneous and tPA-induced recanalisation in the thrombin model.
(A) Representative 2D-TOF images of a longitudinally studied mouse after thrombin-induced MCAo which was not thrombolysed. Whereas the MCA was occluded at +3 hours, it spontaneously recanalized at +24 hours. (B) Mean angiographic score (see supplementary methods section) of longitudinally studied mice after thrombin-induced MCAo (n=4 per group) showing spontaneous and tPA-induced reperfusion in the thrombin model.
Supplementary Figure 2: Binding half-life of MPIOs-αVCAM-1 in a permanent stroke model (FeCl₃): (A) Kinetics of MPIOs-αVCAM-1 clearance from the inflamed vasculature in the FeCl₃ model (24 hours after MCAo, ischemic lesion was assessed on T2-weighted imaging (B)) and corresponding quantification (C) (n=3 per group). Signal voids decreased gradually with time after MPIOs-αVCAM-1 injection. There was no more labeling at +24 hours.
Supplementary Figure 3: Comparison of MPIO-IgG and MPIO-αVCAM-1 induced signal voids in the pMCAO model. Representative T2*-weighted and T2-weighted images of the ischemic brain of mice 20 minutes after either MPIO-IgG (A) or MPIO-αVCAM-1 (B) injection. The orange dotted line represents the ischemic lesion as seen on T2-weighted images. Signal voids corresponding to VCAM-1+ vessels were only present after MPIO-αVCAM-1 injection (particularly at the periphery of the ischemic lesion, yellow arrows), confirming the specificity of the contrast agent used.
Supplementary Figure 4: Inflammatory response differs between permanent and transient MCAo as assessed by MPIOs-αVCAM-1 imaging: (A) Representative images of longitudinal molecular imaging of VCAM-1 revealing VCAM-1 upregulation restricted to the ischemic lesion in the transient model (transient mechanical occlusion of the MCA). VCAM-1 expression gradually decreased during the follow up period. (B) The same as in (A) and but for pMCAo induced by topical application of FeCl₃ on the MCA. VCAM-1 expression was sustained during the follow-up period and particularly significant in the perilesional area. (C) Longitudinal T2-weighted imaging revealed delayed growth of the ischemic lesion between +24h and +72h in the permanent (FeCl₃) but not in the transient (mechanical) MCAo model. (D) Quantification of the MPIOs-αVCAM-1-induced signal void in the ipsilateral cortices at +24h, +72h and +5d after MCAo in the transient mechanical occlusion model and in the FeCl₃ model (n=5 per group). Altogether, these data confirm the findings in the electrocoagulation and thrombin model, suggesting that the differential kinetics in VCAM-1 expression measured by the MPIOs-αVCAM-1 based MRI in the permanent compared to the transient MCAO model is due to the different types of stroke rather than to the method of stroke induction.
Supplementary Figure 5: Numerous activated microglial cells are present in the recruited inflammatory penumbra 72 hours after MCAo: (A) Representative low magnification images of the periphery of the ischemic lesion at 72 hours, when the inflammatory penumbra has been recruited by the ischemic core. Astrocytes (GFAP) were located outside of the MCAo-triggered lesion delineated by a yellow dotted line (as assessed by MAP-2 immunostaining, not shown), whereas numerous microglial cells (Iba1) were located in the periphery of the lesion. (B) Higher magnification images revealed that the intralesional microglial cells display an activated amoeboid shape (small arrows) whereas extralesional microglial cells are ramified (big arrows). These immunohistological findings demonstrated that microglial cells becomes activated in the inflammatory penumbra.
Supplementary Figure 6: Oligemia induces stronger upregulation of VCAM-1 than excitotoxic neuronal cell-death. (A) Left: Schematic representation of the common carotid artery (CCA) ligation model. Right: Representative T2 and T2*-weighted images after MPIO-αVCAM-1 injection 24 hours after CCA ligation. (B) Left: Schematic representation of the intrastriatal administration of N-Methyl-D-Aspartate. Right: Representative T2 and T2*-weighted images after MPIO-αVCAM-1 injection 24 hours after NMDA injection. (C) Mean lesion size as assessed by T2-weighted imaging 24 hours after surgery, revealing the lack of ischemic lesion in the CCA ligation model. In contrast, intrastriatal NMDA administration induced an excitotoxic lesion in the right striatum of all the studied animals (n=4 per group). (D) MPIO-αVCAM-1-induced signal void (in % of the ipsilateral striatum) in the two experimental models. CCA ligation leads to a stronger upregulation of VCAM-1 than ischemia-independent excitotoxic neuronal cell death (n=4 per group).
Supplementary Figure 7: Dipyridamole does not alleviate VCAM-1 expression following pMCAo. (A) Left: Representative images of longitudinal T2-weighted imaging in control mice revealing delayed post-ischemic infarction between 24 and 72 hours. Right: Representative image of MPIOs-αVCAM-1-enhanced imaging 24 hours after MCAo in control mice revealing numerous signal voids in both ipsilateral and contralateral sides, especially in the periphery of the ischemic lesion (B) The same images as in (A) but for dipyridamole-treated mice, revealing an equivalent VCAM-1 overexpression and the persistence of delayed infarct expansion. (C) and (D) Quantification of the MPIOs-αVCAM-1-induced signal void in the ipsilateral (C) and contralateral (D) sides of control and atorvastatin-treated mice (n=3 per group).
Supplementary Figure 8: Influence of tPA-induced thrombolysis on cerebrovascular inflammation. (A) Left: Representative images of longitudinal T2-weighted and T2*-weighted imaging in control mice revealing a large cortical lesion and numerous MPIO-αVCAM-1 induced signal voids in the ischemic lesion. (B) The same images as in (A) but for tPA-treated mice, revealing a smaller cortical lesion. (C) Mean lesion sizes at +24h in control and tPA-treated mice as assessed by T2-weighted imaging (n=5 per group). (D) Quantification of the MPIOs-αVCAM-1-induced signal void in the ischemic lesion of saline- and tPA-treated mice (n=5 per group).
Supplementary Figure 9: Comparison of MPIO-αVCAM-1 induced signal voids following saline and collagenase injection in the striatum. (A) Representative T2-weighted images of the brain of mice 24 hours after either saline or collagenase injection in the right striatum. (B) Representative T2*-weighted images after MPIO-αVCAM-1 administration of the brain of mice 24 hours after either saline or collagenase injection in the right striatum. Confirming our previous results, intrastriatal saline administration induces VCAM-1 expression in small vessels of the ipsilateral side. In contrast, collagenase administration induces a widespread VCAM-1 overexpression, especially in large vessels and in the periphery of the hematoma. The lack of MPIO-αVCAM-1-induced signal voids inside the hematoma could be due to collagenase-induced thrombosis and/or destruction of the vessels.