Ultra-sensitive molecular MRI of cerebrovascular cell activation enables early detection of chronic central nervous system disorders

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Since endothelial cells can be targeted by large contrast-carrying particles, molecular imaging of cerebrovascular cell activation is highly promising to evaluate the underlying inflammation of the central nervous system (CNS). In this study, we aimed to demonstrate that molecular magnetic resonance imaging (MRI) of cerebrovascular cell activation can reveal CNS disorders in the absence of visible lesions and symptoms. To this aim, we optimized contrast carrying particles targeting vascular cell adhesion molecule-1 and MRI protocols through both in vitro and in vivo experiments. Although, pre-contrast MRI images failed to reveal the ongoing pathology, contrast-enhanced MRI revealed hyperperfusion-triggered CNS injury in vascular dementia, unmasked amyloid-induced cerebrovascular activation in Alzheimer’s disease and allowed monitoring of activity during experimental autoimmune encephalomyelitis. Moreover, contrast-enhanced MRI revealed the cerebrovascular cell activation associated with known risk factors of CNS disorders such as peripheral inflammation, ethanol consumption, hyperglycemia and aging. By providing a dramatically higher sensitivity than previously reported methods and molecular contrast agents, the technology described in the present study opens new avenues of investigation in the field of neuroinflammation.

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Introduction

Following injury to the central nervous system (CNS), endothelial cells of the cerebrovasculature become activated and express adhesion molecules such as P/E-selectins, Vascular Cell Adhesion Molecule-1 (VCAM-1), Intercellular Adhesion Molecule-1 (ICAM-1) and Activated Leukocyte Cell Adhesion Molecule (ALCAM) (Rossi et al., 2011). This makes in situ detection of endothelial biomarkers promising to unmask CNS disorders. Recently, micro-sized particles of iron oxide (MPIOs, ≥1 μm) targeting activated platelets or endothelial adhesion molecules have been successfully used to reveal cerebrovascular inflammation following cytokine-induced CNS injury by magnetic resonance imaging (MRI) (McAteer et al., 2007; von Zur Muhlen et al., 2008).

Unfortunately, subsequent reports of MPIOs in clinically relevant models (such as middle cerebral artery occlusion and cerebral malaria) revealed that the sensitivities of such reported contrast agents might be too limited to allow reliable assessment of diseases related to cerebrovascular inflammation (Duffy et al., 2012; Hoyte et al., 2010; Serres et al., 2012; von Zur Muhlen et al., 2008), especially at clinical magnetic field strength. Thus, whether in situ detection of endothelial biomarkers by molecular MRI in vivo could be used to reveal asymptomatic CNS inflammation in clinically relevant models remains uncertain.

Based on these previous studies, we aimed at designing a contrast agent for MRI that would provide the highest sensitivity to reveal CNS disorders by detecting cerebrovascular cell activation. Among the endothelial inflammatory biomarkers, VCAM-1 was selected as the most relevant target because VCAM-1 is less expressed than ICAM-1 and ALCAM by resting endothelial cells (Cayrol et al., 2008) and P/E-selectins are not sustainably upregulated on inflamed brain vessels (Döring et al., 2007). Since previous reports suggested higher sensitivity using micro-sized (MPIOs) rather than nano-sized particles of iron oxide (USPIOs), we first compared the sensitivity of these two approaches. Subsequently, we characterized iron oxide particles targeting VCAM-1 in a mouse model of acute brain inflammation induced by intrastriatal Tumor Necrosis
Factor (TNF) injection and compared the sensitivity of this method to Western blot (WB) and quantitative polymerase chain reaction (q-PCR) to reveal CNS inflammation. Then, we performed in vivo molecular imaging in a set of experimental models (vascular dementia, Alzheimer’s disease (AD) and multiple sclerosis) with a particular emphasis on the interest of VCAM-1 molecular imaging to reveal CNS disorders prior to the appearance of visible lesions by standard MRI analysis. Lastly, we evaluated the sensitivity of contrast-enhanced MRI to reveal the CNS injury triggered by peripheral inflammation, ethanol consumption, hyperglycemia and aging which are known risk factors for CNS disorders.

Materials and methods

Targeting-moiety conjugation to MPIOs

Microparticles of iron oxide (MPIOs; diameter 1.08 μm) with p-toluenesulphonyl reactive surface groups (Invitrogen) were used for peptide conjugation. Purified monoclonal rat anti-mouse antibodies for VCAM-1 (including the previously described clone M/K-2 (McAteer et al., 2007); Southern Biotech and the clone A4(292); AbD-Serotec) or control rat IgG (Jackson ImmunoResearch) were covalently conjugated to MPIOs in borate buffer (pH 9.5), by incubation at 37 °C for 48 h. 40 μg of targeting molecule was used for the coating of 1 mg of reactive MPIOs. MPIOs were then washed in phosphate buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) at 4 °C and stored for 24 h at room temperature, to block the remaining active groups. MPIOs were rinsed in PBS (0.1% BSA) and stored at 4 °C. Except when explicitly mentioned, mice received intravenous injection of 1.0 mg Fe/kg of conjugated MPIOs for contrast-enhanced MRI. Imaging was performed 20 min after particle administration as described above.

Aagarose gels

Different ultrasmall particles of iron oxide (USPIOs, provided by Guerbet, Aulnay-sous-Bois) and MPIOs with concentrations of 300 and 30 nM were embedded in 1.5% w/v agarose gels to limit particle mobility for subsequent MRI analyses. Gels were prepared in 15 mL BD falcon tubes with electrophoresis grade agarose (Invitrogen) dissolved in TAE (40 mM Tris-acetate, 1 mM EDTA) buffer. The iron particles were added and carefully dispersed to form a homogeneous mixture. Samples were left to cool down until solidified to avoid air bubbles. MRI was performed as described below at ambient temperature (20 °C).

Mice

Experiments were performed on male Swiss mice (for stereotoxic injection and intraperitoneal lipopolysaccharide (LPS), d-glucose and ethanol administrations) or on male C57Bl/6 mice (for Unilateral Common Carotid Artery occlusion (UCCAo) model) and multiple sclerosis model): (42 ± 3 g and 30 ± 2 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.

Mice were deeply anesthetized with isoflurane 5% and maintained under anesthesia with 2% isoflurane in a 70%/30% gas mixture (N2O/O2) during surgery. The rectal temperature was maintained at 37 ± 0.5 °C throughout the surgical procedure using a feedback-regulated heating system.

Stereotoxic injection of tumor necrosis factor (TNF) and saline

A unilateral striatal injection of TNF (0.1, 0.5 or 1.0 μg in a volume of 1 μL; PeproTech) or saline was performed after placing Swiss male mice in a stereotoxic 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 (calibrated at 15 mm/μL; assistant ref. 555/5; Hecht, Sondheim-Rhoen, Germany).

Acute inflammatory challenges

Swiss male mice received PBS, lipopolysaccharide from Escherichia coli (LPS, 1 mg/kg, i.p.), d-glucose (3 g/kg, i.p.; leading to a blood glucose of ~500 mg/dL 15 min post injection. Hyperglycemia normalized 1 h after injection) or ethanol diluted in PBS (4 g/kg, i.p.). Molecular imaging of VCAM-1 was performed 24 h after the inflammatory challenge as described above (n = 4 per group).

Unilateral common carotid artery occlusion (UCCAo)

C57Bl6/J male mice were placed on the back with a facemask and maintained under anesthesia. After midline cervical incision, the right common carotid artery (CCA) of C57Bl6/J mice was double-ligated with 5.0 silk sutures to mimic vascular dementia as previously described (Yoshizaki et al., 2008). This model induces chronic oligemia of the hypoperfused cortex, leading to delayed spatial memory deficits and isolated white matter damages. Molecular imaging of VCAM-1 was performed 48 h after carotid artery ligation as described above (n = 4 per group).

Alzheimer’s disease model

APP/P51 mice on a C57Bl6/J genetic background were generated as previously described (Radde et al., 2006). Mice were housed in a standard environment with a 12-h light/12-h dark cycle with free access to food and water. In this model, mice coexpress KM670/671NL mutated amyloid precursor protein and L166P mutated presenilin 1 under the control of a neuron-specific Thy1 promoter element. Cerebral amyloidosis starts at 6–8 weeks of age. Molecular imaging of VCAM-1 was performed at the indicated ages in APP/P51 and wild type littermates (n = 3 per group).

Experimental autoimmune encephalomyelitis (EAE)

EAE was induced in 12-week-old male C57Bl6/J mice via subcutaneous immunization with 200 μg recombinant myelin oligodendrocyte glycoprotein (rMOG 1-125) in an emulsion mixed (volume ratio 1:1) with Complete Freund’s Adjuvant (CFA; Difco Laboratories) containing 500 μg of heat-killed Mycobacterium tuberculosis H37Ra (MBT; Difco) (Kooij et al., 2009). Control (CFA) animals were injected with saline mixed with CFA containing 500 μg of heat-killed MBT. All animals were additionally intraperitoneally (i.p.) injected with 200 ng pertussis toxin derived from Bordetella pertussis (Sigma-Aldrich) in 200 μL saline at the time of, and after 24 h following immunization. Mice were examined daily for clinical signs of EAE and were scored as followed: 0, no disease; 1, limp tail; 2, hindlimb weakness; 3, complete hindlimb paralysis; 4, hindlimb paralysis plus forelimb paralysis; and 5, moribund or dead. MRI was performed in mice (n = 5 per clinical score, CS) with CS of 0 (5–8 days post MOG immunization), CS of 1 (8–13 days), CS of 2 (13 to 18 days), CS of 3 (18 to 23 days) and CS of 4 (23 to 28 days).

Magnetic resonance imaging

Experiments were carried out on a Pharmascan 7 T/12 cm system using surface coils (Bruker, Germany). 3D T2*-weighted gradient echo imaging with flow compensation (GEFC, spatial resolution of 93 μm × 70 μm × 70 μm interpolated to an isotropic resolution of 70 μm) with TE/TR 12.6 ms/200 ms and a flip angle (FA) of 24° was performed to visualize MPIOs (acquisition time = 15 min). For in vitro experiments (15 mL BD falcon tubes with agarose gels), the standard T2*-weighted images resolution was 140 μm
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 h; 4 °C) and cryoprotected (sucrose 20% in veronal buffer; 24 h; 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 mouse anti Aj (clone 4GB; 1:800; Abcam) in veronal buffer (pH 7.4). Primary antibodies were revealed using Fab2 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). Bright field images were acquired without specific staining of the sections. In these conditions, MPIOs appeared as white spherical particles surrounded by a black ring.

Extraction of total RNAs and quantitative real-time RT-PCR

Total RNAs (cortices) were extracted from tissues by using the Nucleospin RNA II kit from Macherey-Nagel. Total RNAs (1 µg) from each sample were reverse-transcribed using the iScript™ Select cDNA Synthesis Kit (BioRad, Marne-la-Coquette, France). The following primer sequences were used: VCAM-1 (forward: AAGAGAACCCAGTTGAGGT; reverse: ACTTAGGAGCCAAAAGGA) and housekeeping gene GAPDH (forward: CCAATGGTCTCGTGG; reverse: GCCCTAGATGCTTCA). PCR reagents were prepared with RNase-free water containing primers and IQ SYBR Green Supermix (BioRad). Assays were run in triplicate on the iCycler iQ real-time PCR detection system (Bio-Rad). Relative mRNA transcription was expressed in arbitrary units using the 2^ΔΔCt method where Ct is the threshold-cycle value.

Western blot

Protein samples (30 µg) were resolved on an 8% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane. Membranes were blocked with 1% BSA (bovine serum albumin, Sigma-Aldrich, L’isle d’Abeau, France) in Tris-buffered saline containing 0.05% Tween-20 and incubated with primary antibodies (Rabbit anti-VCAM-1, 1:500; Santa Cruz Biotechnology, Heidelberg, Germany). After incubation with the peroxidase-conjugated goat anti-rabbit secondary antibody, proteins were visualized with an Amersham ECL Western blotting detection reagents and analysis system (GE Healthcare, France) using ImageQuant™ LAS 4000 camera (GE healthcare, France). After stripping of the PVDF membranes, actin levels were investigated using the primary antibody: rabbit anti-actin (1:2000, Sigma-Aldrich) and the peroxidase-conjugated goat anti-rabbit secondary antibody (1:50 000; Sigma-Aldrich).

Statistical analyses

Results are illustrated as the mean±SEM. Statistical analyses were performed using Kruskal–Wallis (for multiple comparisons) followed by Mann–Whitney’s U-test. Statistical significance was concluded for p<0.05.

Results

MPIOs coupled to A(429) display the highest sensitivity to reveal cerebrovascular inflammation

We first compared in vitro the sensitivity of T1-, T2- and T2*-weighted imagings to reveal different types of iron oxide particles (either ultrasmall (USPIOs, ~20 nm) or microsized (MPIOs, ~1 µm)) embedded in 1.5% agarose gels (Fig. 1). MPIOs and USPIOs were compared using the same concentration of iron for both particles. Based on our results, we selected MPIOs as the contrast carrying particle which would provide the highest sensitivity for in vivo molecular imaging. The high-resolution (70 µm isotropic) 3D T2*-weighted imaging with flow compensation (GEFC) was selected as the most sensitive MRI sequence to detect MPIOs (Fig. 1B), revealing numerous dark dots (signal void) corresponding to the localization of the dispersed MPIOs (Figs. 1A–C).

Subsequently, we coupled several targeting moieties to MPIOs and evaluated the sensitivities of the resulting contrast agents to reveal inflamed endothelium after intrastriatal Tumor Necrosis Factor (TNF) injection in vivo (Figs. 2A and B). Twenty four hours after TNF injection (1.0 µg), intravenous administration of 2 mg/kg VCAM-1-targeted MPIOs induced numerous signal voids in the ipsilateral hemisphere on 3D T2*-weighted imaging. Among the targeting moieties tested, A(429), a rat monoclonal IgG, gave the best results in terms of sensitivity. Control IgG were ineffective. The MPIOs targeting VCAM-1 previously described by McAteer et al. (2007) (coupled to M/K2 clone) induced less signal void than the MPIOs described here, especially in small vessels. Quantitative analysis revealed that using MPIOs coupled to the A(429) clone (hereafter mentioned as MPIOs-αVCAM-1), the volume of signal void was 264% larger than using MPIOs coupled to the M/K2 clone after administration of 1.0 µg TNF (p<0.05, n = 4, Fig. 2B). The contralateral signal voids may come from the large amount of TNF administrated, leading to overexpression of VCAM-1 in both ipsilateral and contralateral hemispheres. This hypothesis is supported by the qPCR and WB data (see below).

Then, we performed a dose–response study (0.25 to 2 mg of iron per kg of body weight) to evaluate the lowest dose of MPIOs needed to reliably evaluate cerebrovascular inflammation (Figs. 2C and D). Based on the results, we selected the dose of 1 mg/kg, which provides an interesting compromise between injected iron dose and imaging sensitivity. Because of their size and autofluorescence properties, both bright field (unstained) and fluorescence microscopy detected MPIOs-αVCAM-1 bound to the VCAM-1 positive endothelium (Fig. 2E) allowing rapid and sensitive histological detections of MPIOs.

MPIOs-αVCAM-1 semi-quantitatively reveal cerebrovascular inflammation

To evaluate the impact of different levels of inflammation on MPIOs-αVCAM-1 induced signal voids, we performed a dose–response study by administrating three different doses of TNF (0.1, 0.5 and 1.0 µg) in the striatum of anesthetized mice. 24 h after TNF administration, we performed molecular imaging of VCAM-1 in parallel of an immunostaining for VCAM-1 (Fig. 3A). As expected, the higher was the dose of TNF, the higher was the VCAM-1 expression at 24 h as assessed by immunohistology of the striatum of the TNF treated mice. Quantitative analysis revealed that the number of VCAM-1 positive vessels was correlated to the injected dose of TNF, ranging from 15 (0.1 µg) to 83 (1.0 µg) vessels per mm² of the striatum section (Fig. 3B).
Accordingly, the volume of MPIOs-\(\alpha\)VCAM-1-induced signal voids was correlated with the injected dose of TNF (from 28% at 0.1 \(\mu\)g to 62% at 1.0 \(\mu\)g of TNF, \(p<0.05\), Fig. 3C).

However, the mean volume of MPIO-induced signal void was not significantly different between the 0.5 \(\mu\)g and the 1.0 \(\mu\)g dose of TNF despite an increased number of VCAM-1 positive vessels (as assessed by immunohistology). These data suggest that when the number of VCAM-1 positive vessels exceeds \(\sim 60/\text{mm}^2\) in a 10 \(\mu\)m thick slice of the studied brain structure, the volume of MPIOs-\(\alpha\)VCAM-1-induced signal void is maximal (saturation). Interestingly, after specific binding to VCAM-1 positive vessels, MPIOs-\(\alpha\)VCAM-1 gradually unbound from the endothelium (Fig. 3D). Their clearance was of 50% after 2 h and was almost complete after 24 h (Fig. 3E).

Current methods to evaluate VCAM-1 expression include whole tissue quantitative polymerase chain reaction (q-PCR) and Western blot (WB). To compare the sensitivity of MPIOs-\(\alpha\)VCAM-1 enhanced MRI to these widely used methods to detect cerebrovascular inflammation, we used paradigms to promote either low-level of inflammation (in situ injection of 1 \(\mu\)L of 0.9% saline) or high-level of inflammation (in situ injection of 1.0 \(\mu\)g of TNF). Interestingly, although MPIOs-\(\alpha\)VCAM-1 enhanced MRI revealed significant ipsilateral overexpression of VCAM-1 in the two conditions tested (Figs. 4A to D), q-PCR and WB revealed increased expression of VCAM-1 only when inflammation raised a high-level (e.g. TNF-treated mice). The fact that qPCR and WB were performed on the entire brain and non-soley on the endothelial cells may explain the low sensitivity of these two methods to reveal VCAM-1 overexpression in the present experimental conditions.

**MPIOs-\(\alpha\)VCAM-1 reveal the inflamed vasculature in a model of vascular dementia**

Unmasking cerebrovascular inflammation in conditions in which classical imaging fails to reveal any parenchymal pathology remains one of the main challenges of molecular imaging. To investigate the interest of MPIOs-\(\alpha\)VCAM-1 to this aim, we performed a previously described murine model of vascular dementia induced by a permanent ligation of the right common carotid artery, which induces chronic hypoperfusion leading to delayed (4 weeks after arterial ligation) white matter lesions and cognitive impairment (Yoshizaki et al., 2008). Forty eight hours after arterial ligation, we evidenced overexpression of VCAM-1 in the ipsilateral cortex by immunostaining in the absence of histological signs of neuronal death (Fig. 5A). Before intravenous MPIOs injection, no abnormality was discernable on T2-weighted imaging (not shown) and 3D T2*-weighted imaging (Fig. 5B). Interestingly, MPIOs-\(\alpha\)VCAM-1 induced strong signal voids from vascular origin in the hypoperfused hemisphere, thus unraveling cerebrovascular inflammation. Control MPIOs-IgG failed to induce signal voids. Quantitative analysis of VCAM-1 positive vessels, revealed a 6-fold increase in the hypoperfused hemisphere (\(p<0.05\)). Similarly, there were 5-folds more MPIOs-\(\alpha\)VCAM-1 bound in ipsilateral (hypoperfused) vessels than in contralateral vessels (\(p<0.05\)) (Figs. 5C to F). Histological studies confirmed the co-localization of MPIOs-\(\alpha\)VCAM-1 and VCAM-1 positive vessels (Fig. 5G).

**MPIOs-\(\alpha\)VCAM-1 unmask cerebrovascular inflammation in a mouse model of Alzheimer’s disease**

Inflammation may play a role in the pathogenesis of several neurodegenerative disorders and represents therefore a potential therapeutic target. Monitoring of inflammation could improve patient selection and therapeutic monitoring in these pathologies. Hence, in another set of experiments, we investigated whether MPIOs-\(\alpha\)VCAM-1 could reveal VCAM-1 upregulation in the brain of APP/PS1 mice, a murine model of Alzheimer’s disease. We performed immunohistological studies of A\(\beta\), Iba-1 and VCAM-1 in the brain of 20 month-old APP/PS1 mice (Fig. 6A). VCAM-1 was overexpressed in APP/PS1 mice in all the brain regions studied (cortex, hippocampus and cerebellum, Figs. 5A and B). Interestingly, a significant cerebrovascular inflammation was detected in the cerebellum of APP/PS1 mice, which was associated with
intravascular Aβ deposition (arrows in Fig. 6A). Of note, there were approximately 3-folds more VCAM-1 positive vessels in the cerebellum than in the cortex of APP/PS1 mice (Fig. 6B). Accordingly, signal voids induced by MPIOs-αVCAM-1 were significantly increased in APP/PS1 mice in all structures compared to age-matched WT mice (signal void areas in % of total structure volume were: 11.85% vs 1.84% (hippocampus), 12.34% vs 3.42% (cortex) and 30.1% vs 4.59% (cerebellum), for APP/PS1 and WT mice, respectively, all p < 0.05, Figs. 6C and D). There were 2-folds more MPIOs-αVCAM-1 induced signal voids in the cerebellum than in the cortex, confirming histological results. As a control, MPIOs-αIgG did not induce any relevant signal void in these mice. Further, immunohistological studies confirmed the MRI findings and revealed frequent co-localization of Aβ with activated microglia, VCAM-1 positive vessels and MPIOs-αVCAM-1 (Fig. 6E).

MPIOs-αVCAM-1 allow early diagnosis in experimental autoimmune encephalomyelitis

Current MRI protocols in patients affected by multiple sclerosis or in experimental autoimmune encephalomyelitis fail to reveal the earliest inflammatory processes of the pathology, especially when the clinical manifestations of the disease are absent. Therefore, monitoring inflammation could provide new insights for diagnosis and monitoring of the disease. Here, we investigated the binding of MPIOs-αVCAM-1 in the CNS of MOG-induced EAE mice according
to their clinical score. We observed a gradual increase in MPIOs-αVCAM-1 induced signal void in the brain and the cerebellum (Figs. 7A and B). In sham animals, signal void area in % of total structure volumes was inferior to 3% in all structures (Fig. 7B), whereas in EAE animals with clinical score of 0 (CS0, asymptomatic), it reached 8.3% and 9.3% in the forebrain and the cerebellum, respectively (both p<0.05). MPIOs-αVCAM-1 binding was maximal in mice with paralyzed hind legs and beginning front limb paralysis (CS4), reaching 24.0% and 40.7% of the total structure volumes, respectively in the forebrain and the cerebellum. Whatever the clinical score, MPIOs-αVCAM-1 induced signal void in both gray matter and white matter. In the cerebellum, MPIOs-αVCAM-1 binding was predominant in the white matter. Frequently, areas of intense MPIOs-αVCAM-1 binding surrounded the corpus callosum of CS4 animals (arrowhead on Fig. 7A). Interestingly, contrast-enhanced MRI revealed EAE pathology before appearance of any symptoms (CS0). Immunohistological studies confirmed the results of the imaging experiments: VCAM-1 expression starts in the brain and the cerebellum and progressively increases in parallel of symptom evolution (Fig. 7C).

**Influence of systemic challenges and aging on cerebrovascular cell activation**

In another set of experiments, we investigated the sensitivity of MPIOs-αVCAM-1 to reveal VCAM-1 overexpression following common inflammatory challenges (peripheral inflammation by intraperitoneal
voids were probably due to the plugging of microvessels by small MPIO aggregates.

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Discussion

In the present study we showed that MPIOs-αVCAM-1 allow non-invasive and sensitive molecular imaging of cerebrovascular cell activation in a set of pre-clinical models of CNS disorders. In all the models performed, CNS injury led to an increased and persistent (>24 h) VCAM-1 expression in the cerebrovasculature without discernible abnormalities on standard imaging. This result suggests that molecular imaging of cerebrovascular cell activation could be used as a reliable and universal marker of CNS injuries. Whether this principle could be extended to all neurological pathologies and vascular beds remains however to be definitely established.

A frequent drawback in molecular MRI protocols is the high amount of material injected (both iron and targeting moiety), precluding repeated administrations of the contrast agent and increasing the cost of the imaging procedure. Interestingly, the iron dose injected in the present study (1 mg/kg) is significantly lower than the doses which have been used in clinical studies involving iron oxide particles (Saleh et al., 2004) (2.6 mg/kg) and the doses reported in mice using other targeted micro-sized particles (Hoyte et al., 2010; McAteer et al., 2004) (4.5 mg/kg). Furthermore, we showed here that the antibody dose needed to reliably evaluate cerebrovascular inflammation could be limited to 0.15 mg/kg and the potency of MPIOs-αVCAM-1 to induce signal changes avoids the necessity to perform pre-contrast scanning. Moreover, complete clearance of MPIOs from the inflamed vasculature reached 24 h after injection, thus allowing repeated imaging experiments and longitudinal assessment of inflammation in chronic pathologies.

Furthermore, the large size of these particles avoids their passive extravasation across injured blood brain barriers (BBB). For the same reason, MPIOs require high-affinity interaction with their targets to prevent their detachment from the vascular wall, avoiding unspecific, low-affinity endothelial binding and providing high specificity of the resulting contrast agent for its target, as confirmed in our control experiments. Some unspecific signal voids may, however, occur in case of microvascular plugging by small MPIO aggregates (Fig. 4). Infiltrating cells may also be responsible for false positive findings, since MPIOs are actively uptaked by the monocyte/macrophage cell line (Wu et al., 2006). Notably, such kind of unspecific monocyte/macrophage labeling was not seen in the experimental conditions of the present study. These unique characteristics strongly support further development of biocompatible MPIO-based contrast agents for clinical use.

Fig. 4. MPIOs-αVCAM-1 reveal cerebrovascular inflammation with ultra-high sensitivity. (A) Expression of VCAM-1 (mRNA) in control mice and in mice that received intrastriatal saline (low-level) or TNF administration (high-level inflammation) 24 h before euthanasia. Overexpression of VCAM-1 was significant only in the high-level inflammation group (n=4 per group). (B) VCAM-1 expression as assessed by western-blot in the low-level and high-level inflammation groups. VCAM-1 upregulation was significant only in the high-level inflammation group (n=4 per group). (C) and (D) Same experiments as in (A) and (B) but VCAM-1 expressions were assessed using MPIOs-αVCAM-1. In both low-level and high-level inflammation groups, contrast-enhanced imaging revealed ipsilateral cerebrovascular inflammation (n=4 per group). Contralateral circular signal voids were probably due to the plugging of microvessels by small MPIO aggregates.

Injection of 1 mg/kg lipopolysaccharide from E. coli (LPS), acute hyperglycemia (3 g/kg) and acute ethanol intoxication (4 g/kg)). Significantly more signal voids were induced by MPIOs-αVCAM-1 in LPS-, D-glucose- and ethanol- than in phosphate buffered saline-treated animals (all r<0.05, Figs. 8A and B). Interestingly, the binding of MPIOs-αVCAM-1 to non-invasively reveal cerebrovascular inflammation associated with aging, a phenomenon known as “inflammaging” (Franceschi and Bonafè, 2003). The high sensitivity of MPIOs-αVCAM-1 to non-invasively reveal cerebrovascular inflammation prompted us to investigate whether the activation level of cerebrovascular endothelial cells increases with aging in mice. To this aim, we compared cerebrovascular inflammation in young (3 months) and aged (24 months) Swiss mice. In aged mice, there were significantly more signal voids induced by MPIOs-αVCAM-1 than in young mice (4.2% vs 11.4% of the ipsilateral hemisphere, respectively, p<0.05, Figs. 8D and E).
Fig. 5. MR molecular imaging of VCAM-1 in a unilateral common carotid artery occlusion model (UCCAO): (A) following right CCA ligation, a marked overexpression of VCAM-1 was observed by immunohistofluorescence in the ipsilateral side at 48 h post-occlusion. Scale bar: 100 μm. (B) Unlike untargeted MPIOs-IgG (lower panel), MPIOs targeted to VCAM-1 (upper panel) unmasked the chronic vascular inflammation seen in this model. Quantification of MPIOs bound to vessels (D), VCAM-1 positive vessels (E) and signal voids area (n=4 per group) were all significantly higher in the hypoperfused hemisphere. (F) VCAM-1 mRNA expression was slightly but not significantly upregulated in this model (n = 4 per group). (G) Representative images of MPIOs targeted to VCAM-1 bound to VCAM-1 positive vessels. Large superficial vessels were characterized by a large amount of bound MPIOs (right panel). JV = jugular vein. CCA = common carotid artery. w/o = without.
Our results suggest that molecular imaging of inflammation using MPIOs-αVCAM-1 can be used to unmask asymptomatic CNS disorders. Hence, in patients with severe carotid stenosis for example, molecular imaging of VCAM-1 may be used to assess whether the chronic cerebral hypoperfusion would lead to CNS damage and thus whether it should be aggressively managed or not. Additionally, in patients with suspected multiple sclerosis, imaging of cerebrovascular cell activation could be useful to confirm widespread cerebrovascular inflammation and thus help to identify early inflammatory process, especially in patient without significant abnormality on conventional MRI. Indeed, in EAE mice, MPIOs-αVCAM-1-enhanced imaging revealed the earliest biological manifestations of the autoimmune processes. Before appearance of symptoms (CS0), VCAM-1 expression was already significantly upregulated in the brain and the cerebellum and then increased gradually and spatially with time and evolution of symptoms.

Moreover, our data suggest that MPIOs-αVCAM-1 allows non-invasive and repeated measurement of cerebrovascular inflammation and thus, could be used to evaluate treatment efficiency in chronic neurological diseases. For example, in EAE mice, endothelial VCAM-1 expression parallels disease activity, suggesting that the therapeutic efficiency of immunomodulatory strategies could be monitored by molecular imaging of VCAM-1. Moreover, in transgenic mice with AD-like amyloid deposits, we demonstrated that endothelial VCAM-1 is upregulated, supporting the link between AD and cerebrovascular inflammation (Tan et al., 2007; Zuliani et al., 2008). Whether anti-inflammatory treatments aiming at slowing down amyloid deposition would reliably reduce cerebrovascular inflammation could be easily investigated using MPIOs-αVCAM-1.

Recently, Serres and co-workers demonstrated similarly that pre-symptomatic lesions in an EAE mouse model can be quantified using VCAM-1-targeted MPIOs (Serres et al., 2011). They used the M/K2 antibody as a targeting moiety, whereas we coupled MPIOs to the A(429) clone. Thus, the MPIOs-αVCAM-1 used in the present study was probably more sensitive to detect cerebrovascular VCAM-1...
expression. This could explain the apparent low level of VCAM-1 expression in the forebrain of pre-symptomatic animals in their study, although they administered a four times higher dose of MPIOs (4 mg/Fe/kg). One other difference between the two studies is the delay between MPIOs injection and imaging. Since we found that MPIOs-αVCAM-1 were rapidly cleared from the circulation, we performed the imaging 20 min after MPIOs injection, whereas Serres and coworkers reported a midpoint of acquisition 1.5 h after MPIOs injection. Therefore, the reduced delay between MPIOs injection and imaging could also have improved the sensitivity of the imaging procedure in the present study.

Our results also further demonstrate the susceptibility of the CNS to systemic inflammatory challenges. Indeed, significant overexpression of VCAM-1 in the CNS was detected not only during chronic neuroinflammatory disorders, but also following acute ethanol consumption, hyperglycemia and peripheral inflammatory challenge, whereas MPIOs-αVCAM-1 binding in the temporal muscle was unmodified (Fig. 8). MPIOs-αVCAM-1 are therefore particularly interesting to study the mechanisms driving the link between systemic inflammation and neuroinflammation, which represent an attractive therapeutic target to prevent reactivation of inflammatory lesions of the CNS (Serres et al., 2009). Additionally, in aged animals, MPIOs-αVCAM-1 enhanced imaging revealed a significantly increased level of cerebrovascular activation. The impact of this cerebrovascular inflammation on aging and senescence remains elusive (Hasegawa et al., 2012) and deserves further investigations.

In conclusion, we report here a molecular imaging contrast agent for MRI which allows ultra-sensitive assessment of cerebrovascular cell activation. By looking at the endothelial cells, diagnosis and non-invasive evaluation of CNS disorders appear to be feasible even before symptom appearance. Since its sensitivity to detect cerebrovascular inflammation compares favorably with q-PCR and WB, the described MPIOs-αVCAM-1 are likely to become a valuable tool to non-invasively and quantitatively evaluate neuroinflammation in pre-clinical models. Moreover, our study provides a basis for further development of endothelium-targeted contrast agents to improve diagnosis accuracy of MRI in patients affected by CNS disorders.

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