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***In vivo* magnetic resonance imaging of acute brain inflammation using microparticles of iron oxide**

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Abstract

Multiple sclerosis (MS) is a disease of the central nervous system that is associated with leukocyte recruitment and subsequent inflammation, demyelination and axonal loss. Endothelial vascular cell adhesion molecule-1 (VCAM-1) and its ligand, $\alpha_4\beta_1$ integrin, are key mediators of leukocyte recruitment and new selective inhibitors that bind to the α_4 subunit of $\alpha_4\beta_1$ substantially reduce clinical relapse in MS. Urgently needed is a molecular imaging technique to accelerate diagnosis, quantify disease activity and guide specific therapy.

We report *in vivo* detection of VCAM-1 in acute brain inflammation, using MRI in a mouse model, at a time when pathology is otherwise undetectable. Antibody-conjugated microparticles carrying a high payload of iron oxide provided potent, quantifiable contrast effects that delineated the architecture of activated cerebral blood vessels. Rapid clearance from blood resulted in minimal background contrast. This technology is adaptable to monitor expression of endovascular molecules *in vivo* in a range of pathologies.

Multiple sclerosis (MS) is a disease of the central nervous system characterized by multifocal white matter lesions¹. Current diagnostic criteria for MS, incorporating both clinical and magnetic resonance imaging (MRI) characteristics, require the demonstration of lesion dissemination in both time and space^{2, 3}. T2-weighted and gadolinium-enhanced T1-weighted MRI detect some, but not all, lesions while advanced MRI techniques such as diffusion imaging⁴, magnetization transfer⁵ and MR spectroscopy⁶ may provide additional insights. However, these approaches are limited in two key respects: (1) they image downstream injury, reflecting relatively advanced pathology and (2) while providing an indication of *severity*, current imaging techniques can not accurately assess disease *activity*⁷.

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R.P.C. and M.A.M. designed the contrast agent. M.A.M. manufactured the contrast agent and, in conjunction with N.W., K.M.C., C.v.z.M and J.S., undertook the *in vitro* experiments. N.R.S., D.C.A., R.P.C and M.A.M. designed the *in vivo* experiments. N.R.S., A.S.L. and D.C.A. conducted the magnetic resonance imaging component and C.v.z.M. and D.C.A. undertook histological analysis. R.P.C supervised image analysis and analysed the data. M.A.M., N.R.S. and R.P.C. contributed to the writing of the manuscript and all authors discussed, and refined the manuscript.

Note: Supplementary information is available on the Nature Medicine website.

There is a pressing need for molecular imaging techniques that can identify early pathogenesis to accelerate accurate diagnosis and guide specific therapy.

Vascular cell adhesion molecule-1 (VCAM-1) and its ligand $\alpha_4\beta_1$ integrin (also termed Very Late Antigen-4, VLA-4) are important mediators of mononuclear leukocyte recruitment and lesion initiation⁸. VCAM-1 is not constitutively expressed on cerebral vascular endothelium, but is up-regulated with endothelial activation⁹. Selective blockade of this interaction in experimental autoimmune encephalitis (EAE, the rodent analogue of MS) results in abolition of lymphocyte recruitment and the paralysis that usually follows¹⁰. Similarly, selective adhesion molecule inhibitors that bind to $\alpha_4\beta_1$ blocks binding to VCAM-1 resulting in substantial reduction in new or enlarging lesions on MRI and in clinical relapse in MS¹¹. We reasoned that the sensitivity of MRI for the detection of early inflammation could be enhanced using a molecular contrast agent directed against VCAM-1. Such an approach could potentially provide more precise and earlier diagnosis and insights into disease activity, prognosis and response to specific therapy.

Micro-particles of iron oxide (MPIO, size range 0.76–1.63 μm) have been used for cellular imaging and tracking¹². For some molecular imaging applications the size of these particles would preclude delivery to the site of interest. However, for imaging endovascular structures, MPIO possess several positive attributes. Firstly, MPIO convey a payload of iron that is orders of magnitude greater than that of the ultrasmall particles of iron oxide (USPIO) that have been used previously for MRI contrast. Secondly, the effects of MPIO on local magnetic field homogeneity, and therefore detectable contrast, extend for a distance approximately 50 times the physical diameter of the microparticle¹². Thirdly, the size of MPIO means that they are less susceptible than USPIO to extravasation or non-specific uptake by endothelial cells and therefore retain specificity for molecular targets¹³.

Accordingly, we have developed a VCAM-1 antibody-conjugated 1 μm MPIO that shows specific and quantitative binding to activated endothelial cells in culture. We report here that this targeted MPIO contrast agent detects VCAM-1 expression *in vivo* in mouse brain inflammation using MRI, with high specificity and exceptional conspicuity.

Results

VCAM-MPIO binds specifically to TNF- α stimulated sEND-1 cells *in vitro*

Monoclonal antibodies against VCAM-1 were conjugated to 1 μm diameter MPIO (termed VCAM-MPIO). We tested the capacity of this construct for specific and quantitative binding *in vitro* on cells of a mouse endothelial line (sEND-1) that were exposed to graded doses of TNF- α , an inflammatory stimulus that was used to provoke surface expression of VCAM-1. After extensive washing, we quantified antibody-MPIO binding under differential interference contrast microscopy. VCAM-MPIO was retained sparsely by unstimulated sEND-1 cells, reflecting low level basal VCAM-1 expression. The number of VCAM-MPIO bound to sEND-1 cells increased in response to increasing doses of TNF- α (Fig. 1a,b). Isotype IgG-1-MPIO negative control constructs did not bind to TNF- α stimulated sEND-1 cells. Further to demonstrate specific retention, we pre-incubated VCAM-MPIO with a chimeric protein containing the extracellular domain of VCAM-1 (Fc-VCAM-1). Blocking with this soluble ligand almost entirely abolished subsequent VCAM-MPIO retention by TNF- α -stimulated sEND-1 cells. By contrast, pre-incubation with soluble extracellular ICAM-1 (Fc-ICAM-1) had no effect on VCAM-MPIO retention, assessed by confocal microscopy (Fig. 1c,d).

Flow cytometry confirmed low basal VCAM-1 expression by sEND-1 cells, which was strongly upregulated with TNF- α (Fig. 2a). Pre-incubation of anti-VCAM-1 antibody with

soluble Fc-VCAM-1 specifically inhibited VCAM-1 binding, while pre-incubation of anti-VCAM-1 antibody with Fc-ICAM-1 had no effect (Fig. 2b,c).

***In vivo* MRI detects binding of VCAM-MPIO to brain endothelium**

To induce endothelial activation and VCAM-1 expression *in vivo*, mice were given interleukin-1 β (IL-1 β) by unilateral stereotaxic micro-injection to the left striatum. We subsequently administered antibody-MPIO constructs systemically by tail vein injection. Circulation time allowed for both specific binding in the brain and clearance of unbound contrast from the blood. Mice underwent MRI, under general anesthesia, 4.5–5.5 h after IL-1 β injection and 1.5–2.0 h after administration of contrast agent.

VCAM-MPIO caused a striking MRI contrast effect manifest as intensely low signal areas that appeared to delineate blood vessels on the IL-1 β -injected side of the brain. Non-specific retention was almost absent from the non-injected hemisphere (**Figs. 3a and 4a and Supplementary video 1**, online). The dynamics of leukocyte-endothelial binding are complex and depend on multiple receptor-ligand interactions. To mimic leukocyte binding more closely, dual antibody-conjugated MPIO were constructed, targeting both VCAM-1 and P-selectin. These dual-conjugated MPIO also bound specifically but did not further enhance contrast effects (Fig. 3b). Control mice that underwent the same injection regime with the substitution of an irrelevant isotype antibody-conjugated MPIO showed no contrast effect (Fig. 3c).

Pre-treatment of animals with anti-VCAM-1 blocking antibody 30 minutes prior to VCAM-MPIO administration abolished retention of VCAM-MPIO despite IL-1 β injection (**Figs. 3d and 4b and Supplementary video 2**, online). Similarly, control animals (with no intracerebral injection or with injection of normal saline vehicle only) that received VCAM-MPIO systemically exhibited no specific contrast effects.

To appreciate the extent and architecture of the contrast effect, segmented areas were rendered to create a 3-dimensional volumetric map of contrast binding that clearly demonstrates the delineation of vascular structures in the IL-1 β -stimulated hemisphere, with almost total absence of binding on the contra-lateral, non-activated side (Fig. 4a). Pre-treatment to block VCAM-1 abolished VCAM-MPIO retention (Fig. 4b).

Quantitative binding analysis

Compared with brains without IL-1 β injection, specific contrast (mean $\pm$ SD $\times 10^{-6} \mu\text{m}^3$) was increased over one hundred-fold (3999 ± 1959 vs. 36 ± 94 ; $P = 0.02$) following administration of VCAM-1-MPIO. No further increase in specific contrast was observed for dual anti-VCAM and anti-P-selectin MPIO (Fig. 4c).

Distribution of MPIO on histology

Histological examination showed VCAM-MPIO lining venules in the IL-1 β stimulated hemisphere (47 ± 15 per section) with sparse retention in the contra-lateral, non-activated hemisphere (3 ± 4 , $P < 0.0001$) (Fig. 5). MPIO were confined to the lumen of the vessel without extravasation. Isolated MPIO were the most common finding. Small clusters, similar to those seen *in vitro* were present, but in relatively small numbers (Fig. 5b,c). Phagocytic cells with MPIO within their cytoplasm were occasionally identified. VCAM-1 expression was confirmed by immunohistochemistry, showing a distribution that was limited to vascular endothelium (Fig. 5b).

Safety and tolerability

Injection of antibody conjugated MPIO was well tolerated in all mice. None of the 16 mice showed signs of ill effect during close observation for up to 5 h post-injection.

Discussion

We report the development and application of a novel molecular imaging probe that identifies VCAM-1 expression in mouse brain *in vivo* using magnetic resonance imaging. The specificity and potency of the contrast effects are dramatic and are derived from a combination of targeted delivery of a high payload of iron oxide to sites of early inflammation and rapid clearance of MPIO from the blood.

The appeals of this approach are multiple. In the context of multiple sclerosis, this technique images a process that occurs early in disease pathogenesis. Unlike existing techniques, the current approach does not require tissue destruction or compromise to the blood brain barrier. Since inhibiting leukocyte-VCAM-1 interaction is of proven clinical benefit¹¹, the ability to image VCAM-1 raises the possibility of targeting treatment to patients with elevated VCAM-1 expression.

Importantly, VCAM-1 participates in other inflammatory conditions including atherogenesis^{14, 15}, transplant rejection¹⁶ and cancer¹⁷, where therapeutic VCAM-1 targeting may also be effective^{18, 19}. More broadly, by modifying the ligand, MPIO-constructs could readily be adapted to image other endovascular targets that are differentially expressed in a broad range of pathologies.

Ultrasound has shown promise for imaging endothelial molecules using targeted microbubbles²⁰ and recently, an enhanced technique using Sensitive Particle Acoustic Quantification (SPAQ) has been applied to measure ICAM-1 and VCAM-1 in rat brain²¹. However, ultrasound techniques are inherently limited by the need for acoustic windows and ultrasound can not reliably penetrate human skull. By contrast, MRI provides exquisite spatial resolution and tissue contrast, without ionizing radiation, making MRI the imaging modality of choice for many brain pathologies and driving the need for molecular MR contrast.

Gadolinium-based contrast agents shorten T1, providing positive contrast on T1-weighted images²². ICAM-1 expression in rat brain has been imaged *ex vivo* using paramagnetic liposomes²³, and we have targeted E-selectin expression in rat brain *in vivo* using a Sialyl Lewis^x moiety conjugated to Gd-DTPA²⁴. However, the limited quantity of gadolinium that can be delivered to an endothelial monolayer, limits its contrast effect. By comparison, USPIO provide greater contrast, but may require sophisticated ligands that mediate internalization by endothelial cells in order to achieve adequate local concentrations²⁵. USPIO have become popular owing to their long blood half-life, which is a positive attribute for applications such as the measurement of changes in cerebral perfusion. However, this property is more of a hindrance in targeted contrast agents since it leads to high background contrast for an extended period. A further potential drawback of USPIO is that contrast is manifest in T2*-weighted images as indistinct areas of low signal that can be difficult to distinguish from the ordinary heterogeneity of normal tissue. Furthermore, since USPIO can be taken up non-specifically by endothelial cells there is potential to compromise the specificity of molecular targeting¹³.

MPIO convey a payload of iron that is orders of magnitude greater than USPIO and cause a local magnetic field inhomogeneity extending for a distance approximately 50 times the physical diameter of the particle¹². Although smaller than leukocytes and, therefore, not

prone to small vessel plugging, the size and incompressible nature of MPIO preclude translocation across the endothelium, as confirmed by histology. The pattern of MPIO binding appeared almost identical to the patterns of lymphocyte binding in venules in the rat experimental autoimmune encephalomyelitis model of multiple sclerosis¹⁰. Recent trials have demonstrated clear clinical benefits from inhibition of the interaction between VCAM-1 and its ligand¹¹. The ability to image VCAM-1 expression, in conjunction with existing diagnostic approaches, may offer clinically important opportunities to enhance specificity, and accelerate diagnosis. Early diagnosis and delivery of specific guided intervention may improve outcomes and allow response to treatment to be more precisely monitored.

Our approach uses commercially available reagents to provide a generic platform technology for endovascular molecular MRI and potentially allows the substitution of alternative ligands. In respect of translation to clinical use, we identify three key factors: (1) *MPIO size*. At the doses used reported here, short term ill effects were not seen in mice, nor was there evidence of tissue infarction due to small vessel 'plugging'. Indeed *in vivo* imaging and subsequent histological analysis of non-injected hemispheres both confirm that non-specific MPIO retention was minor. (2) *MPIO composition*. The MPIO used here are non-biodegradable and are not suitable for use in humans. However, iron-oxide containing contrast media are already in clinical use and it should be feasible to synthesize biodegradable particles^{26, 27}. (3) *Iron dose*. The iron dose (4.5 mg iron / kg body weight) reflects closely the iron dose of 2.6 mg / Kg that has been used extensively for human oncological MRI using USPIO²⁸ and is considerably less than that used (30 mg / Kg) in a recent study targeting USPIO to image VCAM-1 in mice²⁵.

In conclusion, this novel molecular imaging approach manifests exceptionally potent contrast effects on MRI. In a mouse model of acute inflammation, cerebral blood vessels were delineated and VCAM-1-MPIO binding quantified. Alternative ligand-MPIO constructs provide clear opportunities for diagnostic imaging using specific endothelial cell markers that are differentially expressed in a broad range of pathologies including inflammatory diseases, cancer and atherothrombosis.

Methods

Antibody conjugation to iron oxide microparticles

We used myOne™ tosylactivated MPIO (1 µm diameter: iron content 26%) with p-toluenesulphonyl (tosyl) reactive surface groups (Invitrogen) for antibody conjugation. We washed MPIO with sodium borate buffer (0.1 M, pH 9.5) and added purified monoclonal rat antibodies to mouse VCAM-1 (clone M/K2, Cambridge Bioscience) or IgG-1 (clone Lo-DNP-1, Serotec) or a combination (50%: 50%) of VCAM-1 and P-selectin (clone RB40.34, Fitzgerald Industries) (1×10^9 MPIO per 40 µg antibody for all). We added 3 M ammonium sulphate to give a final concentration of 1 M. We incubated the solution with constant rotation at 37°C for 20 h. After incubation, we pelleted MPIO using a Dynal magnet (Invitrogen) and discarded the supernatant containing any unbound antibody. We added PBS (0.5% BSA and 0.05% tween 20) (pH 7.4) and incubated MPIO at 37°C overnight, to block remaining active tosyl sites. We washed MPIO with PBS (0.1% BSA and 0.05% tween 20) at 4°C before storing at a concentration of 2.5×10^{10} MPIO per ml PBS (0.1% BSA and 0.05% tween 20) at 4°C. We calculated that the primary amine and sulphhydryl groups of 1 µm tosylactivated MPIO have a capacity to bind covalently 1.8×10^9 IgG molecules per MPIO.

Cell culture

Please see **Supplementary Methods**.

Animal protocol

After anesthesia with isofluorane (2.0–2.5% in 70% N₂O: 30% O₂), we placed adult male NMRI mice (35 ± 2 g) in a stereotaxic frame under a Wild M650 operating microscope (Leica). Using a < 50 µm-tipped glass pipette, we stereotactically injected 1 ng of mouse recombinant IL-1β in 1 µl low endotoxin saline into the left striatum (co-ordinates from Bregma: anterior 0.5 mm, lateral 2 mm, depth 2.5 mm), to induce endothelial activation. After 3.1 ± 0.2 h, we injected mice via a tail vein with one of (a) VCAM-1 MPIO, (b) VCAM-1 + P-selectin MPIO, or (c) IgG-1 MPIO (4 × 10⁸; approx. 4.5 mg iron per kg body weight) (*n* = 3 per group). We had two control groups of mice (*n* = 2 per group); one group we injected mice intracerebrally with 1 µl low endotoxin saline and the other group we did not inject intracerebrally. We subsequently administered VCAM-1 MPIO intravenously to both control groups. To determine selectivity of VCAM-1 MPIO, we injected a further group of mice (*n* = 3) with 0.2 mg / Kg VCAM-1 antibody 3.3 ± 0.4 h after intracerebral IL-1β injection, to block VCAM-1 binding sites. We subsequently administered VCAM-1 MPIO 15 min later. Following MPIO injection, we placed the mice in a quadrature birdcage coil with an in-built stereotaxic frame for imaging. We maintained anesthesia using 1.5–1.8% isofluorane in 70% N₂O:30% O₂, monitored ECG and maintained body temperature at ~37°C with a circulating warm water system. All procedures were approved by the UK Home Office.

In vivo magnetic resonance imaging

We used a 7 Tesla horizontal bore magnet with a Varian Inova spectrometer (Varian) to acquire a T₂*-weighted 3D gradient-echo dataset using the following parameters; flip angle 35°, repetition time = 50 ms, echo time = 5 ms, field of view 22.5 × 22.5 × 31.6 mm, matrix size 192 × 192 × 360, two averages, total acquisition time ~1 h. The mid-point of acquisition was 1.7 ± 0.5 h after MPIO injection. We serially imaged the same animal and found maximal contrast between one and two hours with diminution by four hours (data not shown). We zero-filled the data to 256 × 256 × 360 and reconstructed off-line, giving a final isotropic resolution of 88 µm³.

MR image analysis

For each MR image, we masked manually the brain to exclude extra-cerebral structures. We segmented low signal areas in 41 contiguous images, spanning a depth of 3.6 mm from the dorsal hippocampus ventrally. To control for minor variations in absolute signal intensity between individual scans, we calibrated low signal areas on ten evenly spaced slices per brain. We applied the median signal intensity value to the fully automated, histogram-based batch analysis of the 41 slice sequence. We extracted data for left and right sides of the brain simultaneously, with identical parameters. We summated voxel volumes and expressed them as raw volumes in µm³ without surface rendering or smoothing effects. To ensure true laterality, we quantified contrast in each hemisphere 1 mm from the midline outwards. We used ImagePro Plus (Media Cybernetics) to segment and quantify contrast volume by an operator blinded to the origin of all data. We present the data as 'specific contrast', defined as 'left' minus 'right' contrast volume.

Histology

Please see **Supplementary Methods**.

Statistical analysis

We expressed the data as mean $\pm$ S.D. and compared, where indicated, using two tailed t-tests. We assigned statistical significance at $P < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

MPIO binding to cultured sEND-1 cells. (a) Following stimulation with TNF- α . (0 – 10 ng / ml), cells were exposed to VCAM-MPIO or irrelevant isotype-MPIO. In the absence of TNF- α , there was minimal VCAM-MPIO retention by sEND-1 cells. Scale bar = 10 μ m. (b) Dose dependent retention of VCAM-MPIO in response to incremental doses of TNF- α ($R^2 = 0.94$, $P < 0.01$). Binding persisted after extensive washing and was restricted to cellular areas. (c) Confocal microscopy of sEND-1 cells that were stimulated with TNF- α . (50 ng / ml). Green fluorescence reflects VCAM-1 expression on the cell surface. Prior incubation of VCAM-MPIO with soluble extracellular ICAM-1 (Fc-ICAM-1) had no effect on VCAM-MPIO binding (autofluorescent yellow-green spheres, arrows), while preincubation with Fc-VCAM abolished VCAM-MPIO retention by sEND-1 cells almost completely, despite demonstrable surface VCAM-1 expression. sEND-1 cell nuclei stain blue. Scale bar = 5 μ m. (d) Means $\pm$ standard deviation of retained VCAM-MPIO after TNF- α stimulation with and without pre-incubation with soluble Fc-VCAM-1 or Fc-ICAM-1. * $P < 0.0001$

Figure 2.

Flow cytometry. (a) basal low level VCAM-1 expression by sEND-1 cells with marked up-regulation in response to TNF- α . (b). Fc-VCAM-1 potently and specifically inhibited the interaction of VCAM-1 antibody with sEND-1 cells, while Fc-ICAM-1 had no effect. (c) Quantitative analysis confirmed absence of non-specific basal binding of IgG (labeled IgG) and in the presence of TNF- α (IgG TNF- α^+) and low level VCAM-1 expression under basal conditions (labeled VCAM) with marked up-regulation after TNF- α (VCAM TNF- α^+). Antibody binding to VCAM was almost entirely abrogated by Fc-VCAM, while Fc-ICAM had no effect. * $P < 0.001$

Figure 3.

In vivo T_2^* -weighted coronal images (in rows, 4 images per brain) from 3D gradient echo data sets each with $\sim 90 \mu\text{m}$ isotropic resolution. (a). animal injected intrastratially with 1 ng IL-1 β in 1 μl saline 3 h prior to intravenous injection of VCAM-MPIO ($\sim 4.5 \text{ mg iron / kg}$ body weight). Intense low signal areas (i.e. black) on the left side of the brain reflect the specific retention of MPIO on acutely activated vascular endothelium with virtually absent contrast effect in the contra-lateral control hemisphere. (b) similar, unilateral MPIO contrast effects in an animal injected intrastratially with 1 ng IL-1 β in 1 μl saline 3 h prior to intravenous injection of anti-VCAM-1 + anti-P-selectin-MPIO (4.5 mg iron / kg body weight). (c) Absence of MPIO effects in an animal injected intrastratially with 1 ng IL-1 β in 1 μl saline 3h prior to intravenous injection of IgG1-MPIO control (mg iron / kg body weight). (d) absence of MPIO effects in an animal injected with 1 ng IL-1 β in 1 μl into the striatum and with VCAM-MPIO intravenously (4.5 mg iron / kg body weight), but after pretreatment with anti-VCAM-1 antibody, which effectively blocked VCAM-MPIO binding sites. In all cases MRI data were obtained 1 – 2 h post-MPIO injection. Scale bar = 5 mm.

Figure 4.

3-dimensional volumetric maps of VCAM-MPIO binding (red) and quantitative analyses of MPIO contrast effects. (a) In each animal, 41 contiguous images were segmented using an automated analysis of signal intensity histograms. MPIO contrast effects delineated the architecture of cerebral vasculature in the IL-1 β -stimulated hemisphere (image left) with almost total absence of binding on the contra-lateral, non-activated side. The midlines are indicated by vertical sections. (b) Pre-administration of anti-VCAM-1 antibody abolished anti-VCAM-MPIO retention. (c) Compared to brains without IL-1 β injection, specific contrast was increased over one hundred-fold following administration of VCAM-1-MPIO. Dual conjugated MPIOs targeting both VCAM-1 and P-selectin also bound specifically but did not further enhance contrast effects. Substitution of isotype-MPIO (IgG / IL-1 β^+), sham intracerebral injection (VCAM / NaCl), no intracerebral injection (VCAM / IL-1 β^-) or pre-blocking (VCAM / IL-1 β^+ with Block) were not associated with specific contrast effects. Bars indicate mean values for each group. * $P = 0.02$.

Figure 5.

Post mortem light micrographs of mouse brain. **(a)** cresyl violet staining shows MPIO lining a venule on the injected side of the brain. MPIO were confined to the lumen of the vessel (thin arrows). Binding most often comprised isolated MPIO. **(b)** Occasionally clusters of MPIO were seen (clear arrow), some of which appeared to reflect phagocytic cells with MPIO within their cytoplasm. VCAM-1 expression (brown) was confirmed by immunohistochemistry, showing expression limited to vascular endothelium (large arrows) with co-localization of VCAM-1 immunostaining and *in vivo* VCAM-MPIO binding. **(c)** Quantification of MPIO binding in the IL-1-stimulated (IL-1 β ⁺) versus unstimulated hemispheres of mice receiving VCAM-MPIO demonstrated retention of MPIO in the former with a strong preponderance of single MPIO (sMPIO) with less frequent clusters (cMPIO). * $P < 0.0001$. Scale bar = 10 μ m