Magnetic Resonance Imaging of Endothelial Adhesion Molecules in Mouse Atherosclerosis Using Dual-Targeted Microparticles of Iron Oxide

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Objective—Microparticles of iron oxide (MPIO) distort magnetic field creating marked contrast effects far exceeding their physical size. We hypothesized that antibody-conjugated MPIO would enable magnetic resonance imaging (MRI) of endothelial cell adhesion molecules in mouse atherosclerosis.

Methods and Results—MPIO (4.5 μm) were conjugated to monoclonal antibodies against vascular cell adhesion molecule-1 (VCAM–MPIO) or P-selectin (P-selectin–MPIO). In vitro, VCAM–MPIO bound, in dose-dependent manner, to tumor necrosis factor (TNF)-α stimulated sEND-1 endothelial cells, as quantified by light microscopy ($R^2=0.94$, $P=0.03$) and by MRI ($R^2=0.98$, $P=0.01$). VCAM–MPIO binding was blocked by preincubation with soluble VCAM-1. To mimic leukocyte binding, MPIO targeting both VCAM-1 and P-selectin were administered in apolipoprotein E−/− mice. By light microscopy, dual-targeted MPIO binding to endothelium overlying aortic root atherosclerosis was 5- to 7-fold more than P-selectin–MPIO ($P<0.05$) or VCAM–MPIO ($P<0.01$) alone. Dual-targeted MPIO, injected intravenously in vivo bound aortic root endothelium and were quantifiable by MRI ex vivo (3.5-fold increase versus control; $P<0.01$). MPIO were well-tolerated in vivo, with sequestration in the spleen after 24 hours.

Conclusions—Dual-ligand MPIO bound to endothelium over atherosclerosis in vivo, under flow conditions. MRI may provide a functional MRI probe for detecting endothelial-specific markers in a range of vascular pathologies. (Arterioscler Thromb Vasc Biol. 2008;28:77-83.)

Key Words: microparticles of iron oxide ■ atherosclerosis ■ magnetic resonance imaging ■ molecular imaging

Magnetic resonance imaging (MRI) has demonstrated substantial utility in phenotyping vascular disease. Using inherent physico-chemical properties that confer particular tissue relaxivities, it has been possible to characterize the vessel wall in atherosclerosis at a submillimeter level.1,2 However, to capitalize fully on the diagnostic potential of MRI requires imaging at molecular and cellular levels.3-9 To achieve this, purpose-built contrast agents are needed that can identify molecules of interest with high specificity, while conveying sufficient contrast to be easily distinguished from unenhanced tissue.

Specificity can be achieved through conjugation of contrast agent with monoclonal antibodies or their immunospecific fragments F(ab), peptides, or peptide-mimetics. Previous approaches have included integrin-conjugated gadolinium-rich perfluorocarbon nanoparticles,10 peptide-conjugated nanoparticles of iron oxide,11 and fibrin-specific cyclic peptide labeled with gadolinium.12

Gadolinium-based contrast agents shorten T1 providing positive contrast on T1-weighted images. However, for the quantities that can be delivered to an endothelial monolayer, the contrast effects are relatively modest. By comparison, iron oxide nanoparticles provide greater contrast effects, but require many particles to be delivered to a given voxel. Another potential drawback is that contrast effects are manifest in $T_1$-weighted images as dark signal areas that can be difficult to distinguish from surrounding tissue.

Recently, larger microparticles of iron oxide (MPIO) have been used for cellular imaging and tracking.13,14 For some applications the size of these particles would preclude delivery to the site of interest. However, for imaging endo-vascular targets, MPIO possess several positive attributes. Firstly, MPIO convey a payload of iron that is orders of magnitude greater than ultrasmall particles of iron oxide (USPIO). Secondly, the effects of MPIO on local magnetic field homogeneity, and therefore detectable contrast, extend a distance at least 50 times their physical diameter.13 Thirdly, once bound to endothelium, MPIO remain intravascular thereby allowing bound MPIO to be readily distinguished
from the vessel wall. Finally, conjugated MPIO may offer a
generic tool for imaging endothelial-specific markers across
a range of vascular pathologies such as inflammation, athero-
sclerosis, and angiogenesis.

In animal models of atherosclerosis, vascular cell adhesion
molecule-1 (VCAM-1; CD106) mediates adhesion, rolling,
and tethering of mononuclear leukocytes and facilitates their
transmigration to the developing atherosclerotic plaque.15–17
VCAM-1 expression is not constitutive, but is present at
atherosclerosis-prone sites, even before macroscopic disease
is apparent, with persistent expression in more advanced
lesions.16,18 VCAM-1 is therefore a potentially useful marker
for atherosclerosis from early stages. However, in vivo
molecular imaging of the vascular endothelium presents
particular challenges since the contrast agent has to bind in
sufficient density to the target, a two-dimensional monolayer,
that is exposed to significant physiological shear stresses. The
dynamics of leukocyte:endothelial binding are also complex
and dependent on multiple receptor-ligand interactions. Initial
leukocyte rolling is selectin-mediated, whereas firm adhesion
is mediated by integrin binding to intercellular adhesion
molecule-1 (ICAM-1) and VCAM-1, with the latter more
important in initiation of atherosclerosis. “Adhesion dynamic
modeling” predicts synergistic roles for selectins and inte-
grins with transition between rolling and firm adhesion
dependent on binding affinities and relative concentrations of
receptor-ligand interactions.19,20

Accordingly, we adopted a dual antibody-conjugated MPIO
approach for targeted MRI and applied this to the detection of
adhesion molecules on the arterial endothelium of apoli-
oprotein E–knockout (apoE−/−) mice.

Materials and Methods

Antibody Conjugation to Iron Oxide Microparticles

MPIO (4.5 μm diameter) with p-toluenesulphonyl (tosyl) reactive
surface groups (Invitrogen) were used for antibody conjugation.
These tosyl groups react covalently with primary amino and
sulphydryl groups predominantly in the Fc-region of antibodies,
favoring optimal orientation. Purified monoclonal rat anti-mouse
antibodies for VCAM-1 (clone M/K2) (Cambridge Bioscience
Ltd), P-selectin (clone RB40.34), (Fitzgerald Industries Intl), or
IgG-1 (clone Lo-DNP-1; Serotec) were covalently conjugated to
MPIO, by incubation at 37°C for 20 hours, with constant rotation
(1×10⁶ MPIO per 5 μg antibody). Dual-targeted MPIO, incubated
with VCAM-1 and P-selectin antibodies (2.5 μg of each), were
prepared in the same way.

MPIO were then washed twice in phosphate buffered saline (PBS)
containing 0.1% bovine serum albumin (BSA) at 4°C and incubated
with tris buffer (0.1 mol/L, 0.1% BSA, pH 7.4) for 4 hours at 37°C,
to block remaining active tosyl sites. MPIO were rinsed in PBS
(0.1% BSA) at 4°C for 5 minutes and stored at 4°C.

In Vitro Binding of Anti–VCAM-1-MPIO to
TNF-α–Stimulated sEND-1 Cells

Cells of a murine endothelial line (sEND-1) were cultured in
Dulbecco Modified Eagle medium (Invitrogen) supplemented
with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100
U/mL penicillin and 0.1 mg/mL streptomycin. Cells (8×10⁴ per
35 mm well) were incubated with 0.1, 1, 10, or 50 ng/mL murine
recombinant TNF-α (R&D systems) for 20 hours at 37°C to
induce endothelial VCAM-1 expression. Stimulated cells were
incubated in duplicate with VCAM–MPIO or control IgG-1-
MPIO (1.2×10⁶ per 2 mL media) for 30 minutes at RT, with
constant mixing. Unbound MPIO were removed by extensive
washing with PBS.

MPIO binding to cells was assessed in 4 fields of view per TNF-α
dose by light microscopy (Leica DM R; ×20 objective) and
quantified using ImagePro plus (Media Cybernetics).

MRI of Cell Phantoms

Resuspended sEND-1 cells were embedded in 2% high grade
agarose. MRI was performed using an 11.7 Tesla vertical magnet
(Bruker) and 40-mm probe. A 3D gradient echo sequence was used
(TR/TE=4/90 ms, field of view [FOV] 30×30×30 mm, matrix size
[512]², 2 averages, imaging time ~13 hour overnight) with final
resolution of 29.3x29.3x29.3 μm after image reconstruction. Low
signal areas, corresponding to MPIO binding, were quantified in 8
images per sample (at slice intervals of 293 μm) using a histogram
based tool in ImagePro Plus.

ApoE−/− Mice

Homozogous apoE−/− mice, bred in a pathogen-free room, with
constant temperature and humidity, were weaned at 3 weeks of age
and transferred to Western diet (21% milk fat, 0.15% cholesterol:
100244 Dyets Inc) for 25 weeks. Experiments were performed
according to UK Scientific Procedures Act (1986).

Left Ventricular Injection of MPIO

ApoE−/− mice were terminally anesthetized by isofluorane inha-
lation. The chest cavity was exposed and while the heart was
beating, mice were injected via the left ventricle with P-selectin-
MPIO (n=4 mice), VCAM–MPIO (n=7 mice), control IgG-1–
MPIO (n=3 mice), or dual-ligand MPIO targeting P-selectin and
VCAM-1 (n=6 mice) (1.2×10⁶ MPIO in 100 μL PBS; ~6 mg
iron/kg body weight). After 5 minutes, the arterial tree was
perfusion fixed via the left ventricle with 10 mL paraformalde-
hyde (4% in PBS).

In Vivo Systemic Administration of
Dual-Targeted MPIO

ApoE−/− mice were anesthetized with Hypnorm (25 mg/kg, Bayer)
and Hypnoval (25 mg/kg, Bayer) combined, administered subcuta-
nously. Dual-targeted MPIO or control IgG-1-MPIO (6×10⁶ MPIO/
150 μL PBS; ~30 mg iron per kg body weight) were injected in vivo
via the jugular vein (n=4 mice per group) and allowed to circulate
for 30 minutes. Mice were terminally anesthetized and the arterial
tree perfusion fixed via the left ventricle with 4% PFA, removed en
bloc and embedded in a glass MR tube containing 2% agarose.21 The
heart was instilled with 4% PFA containing 2 mmol/L gadoteridol
(Prohance, Bracco) via the left ventricle and the tube sealed with 2%
agarose containing 2 mmol/L gadoteridol.

MRI Analysis

High resolution MRI of the arterial tree was performed ex vivo at
11.7 T using a 13 mm BIRD cage radiofrequency coil (RAPID
Biomedical). A 3D gradient echo sequence was used (TE=4 ms/
TR=90 ms, FOV 13×13×19.5 mm, matrix size 256×256×384,
two averages, imaging time ~7 hours) with final resolution of
25×25×25 μm after data reconstruction. Segmentation of bound
MPIO was performed by an observer blinded to the MR image
identities. MPIO binding was defined as a discrete circular low
signal area on the luminal surface of aortic root in ≥2 consecutive
MR images. MPIO appearing in multiple consecutive images were
counted only once. Segmented images were 3D reconstructed using
the 3D Constructor plug-in for ImagePro Plus to visualize MPIO
contrast throughout the aortic root.

Statistical Methods

Data are mean±SD. Quantification of cell-bound MPIO assessed by
MRI and light microscopy were related to the dose of TNF-α using
simple linear regression (GraphPad Prism 4, GraphPad Software Inc). Parametric data were compared using Student t tests, with statistical significance attributed at probability values <0.05.

Competitive Inhibition of VCAM–MPIO Binding In Vitro
Please see supplementary methods, available online at http://atvb.ahajournals.org.

Assessment of In Vitro Macrophage and sEND-1 Cell Uptake of MPIO by MRI
Please see supplementary methods.

Histology
Please see supplementary methods.

Assessment of MPIO Uptake by Organs Following In Vivo Administration
Please see supplementary methods.

Results

VCAM–MPIO Bind to TNF-α–Stimulated sEND-1 Cells
Assessed by light microscopy, VCAM–MPIO bound to TNF-α–stimulated sEND-1 cells, but did not bind to unstimulated cells. IgG-1-MPIO did not bind to TNF-α–stimulated sEND-1 cells. The number of cell-bound VCAM–MPIO increased with increasing doses of TNF-α (Figure 1A, \( R^2 = 0.94, P = 0.03 \)). High-resolution MRI of cell phantoms showed punctate low signal areas representing VCAM–MPIO binding to cells, which increased in a similar fashion to increasing dose of TNF-α (Figure 1B, \( R^2 = 0.98, P = 0.01 \)). As a demonstration of specificity, preincubation of VCAM–MPIO with soluble Fc–VCAM-1 inhibited anti–VCAM-1-MPIO binding almost entirely. By contrast, preincubation of VCAM–MPIO with soluble Fc–ICAM-1 had no effect (supplemental Figure I).

Assessment of MPIO Uptake by Macrophages and sEND-1 Cells
Mouse peritoneal macrophages and sEND-1 cells were incubated with unconjugated MPIO in vitro. By light microscopy, macrophages phagocytosed MPIO after 30 minutes, and more so by 2 hours. By contrast, there was no MPIO uptake by sEND-1 cells (Figure 2C). With high resolution MRI, discrete low signal circular areas were observed for macrophage cell phantoms preincubated with MPIO for 30 minutes which increased further by 2 hours (\( P = 0.004 \); Figure 2). The equivalent quantification for sEND-1 cells incubated with MPIO for 30 minutes or 2 hours showed no difference from sEND-1 cells alone, indicating absence of uptake.

Single Versus Dual-Targeted MPIO Binding in Mouse Atherosclerosis
VCAM–MPIO and P-selectin–MPIO bound specifically to endothelium overlying atherosclerotic plaque after left ventricular injection in terminally anesthetized mice. The number of VCAM–MPIO and P-selectin–MPIO bound per aortic root section was similar (16.1 ± 2.7 versus 12.9 ± 4.2) with minimal nonspecific retention of IgG-1–MPIO (1.7 ± 4.2; Figure 3). Dual-targeted MPIO showed significantly enhanced binding efficiency compared with P-selectin–MPIO (6.9 fold increase; \( P = 0.027 \)) and VCAM–MPIO (5.5 fold increase; \( P = 0.005 \)).
In Vivo Dual-Targeted MPIO Binding to Atherosclerosis: Quantification With Ex Vivo MRI

Dual-targeted MPIO or IgG-1–MPIO were injected in vivo via the jugular vein and allowed to circulate for 30 minutes. High-resolution ex vivo MRI detected discrete circular low signal areas on the luminal side of the aortic root, in areas overlying atherosclerotic plaques. The contrast effect extended over ~8 voxels. The appearance of MPIO changed in a consistent and characteristic fashion, and varied according to their position within a voxel (Figure 4A). The number of dual-targeted MPIO binding, determined by MRI, was 3.5-fold higher compared with IgG-1–MPIO (14 ± 4.2 versus 4 ± 1.8; P < 0.01; Figure 4B). 3D reconstruction of segmented images demonstrated that MPIO binding was localized to atherosclerotic plaque endothelium throughout the aortic root. No MPIO binding was observed in atherosclerosis-free areas of the ascending aorta (Figure 4C).

Histological Assessment of Organs After In Vivo MPIO Administration

All mice tolerated MPIO injection and none showed signs of ill health up to 24 hours postinjection. MPIO were identified in lungs, kidneys, liver, and spleen after 30 minutes and 24 hours. Importantly, there was no evidence of infarction, hemorrhage, edema, inflammation, or MPIO extravasation in any of these organs, at either time point. Compared with 30 minutes, at 24 hours postinjection there was significant clearance of MPIO from the lungs (78% decrease; P < 0.001) and kidneys (65% decrease; P < 0.001) with redistribution of MPIO predominantly to the spleen (305% increase; P < 0.001) and to a lesser extent, the liver (69% increase; P < 0.01; Figure 5).

Discussion

The activated endothelium displays numerous molecules upregulated in vascular disease providing potential markers for functional molecular imaging and targeted therapeutics. An important challenge for in vivo molecular imaging is to deliver a potent contrast agent in sufficient quantity under conditions of shear stress. Here we combine intense MR contrast effects attainable using microparticles of iron oxide with a dual ligand-targeting strategy modeled on leukocyte interactions with activated endothelium.

Recently, MPIO have been used for imaging inflammatory cells in transplant rejection and for tracking single cells during embryogenesis. The accessibility of the endothelium to intravascular contrast agents permits the use of MPIO that are orders of magnitude larger than USPIO used previously for similar applications. We report the use of targeted MPIO that specifically bind to adhesion molecules in mouse athero-
sclerosis after in vivo administration and which are readily identified by ex vivo MRI.

Dual antibody-conjugated MPIO, targeting both P-selectin and VCAM-1, were developed to mimic in vivo dynamics of leukocyte binding to endothelium. Initial leukocyte rolling, which is selectin-mediated, can be simulated under flow conditions using colloidal microspheres coated with sialyl Lewis^x or P-selectin glycoprotein ligand-1.22 Firm adhesion is mediated by integrin binding to ICAM-1 and VCAM-1. As predicted by computed models, dual-targeted MPIO showed synergistically enhanced binding compared with either in isolation. A similar approach targeting ultrasound microbubbles to vascular endothelium used a combination of sialyl Lewis^x and ICAM-1 antibody, though binding efficiency was increased only marginally, possibly because of stearic limitation from size mismatch between the 2 ligands.23

The role of VCAM-1 in the pathogenesis of atherosclerosis is well established in animal models, where it has been shown to be important in monocyte recruitment.15,18 In humans, Davies et al found VCAM-1 expression in a majority of human coronary atherosclerotic plaques post mortem24 though this is not always consistent.25

High resolution ex vivo MRI showed that, after intravenous injection, dual-targeted MPIO localized rapidly and specifically to aortic root plaque endothelium, a characteristic site for atherosclerosis development in mice. Although MPIO binding was not confluent, the “bloom effect” of magnetic field distortion of MPIO was clearly identifiable as circular low signal areas within the vessel lumen. We did not observe binding to atherosclerosis-free segments of the ascending aorta. Earlier approaches to endothelial molecular MRI have been limited by a lack of sensitivity.26,27 To address this, Nahrendorf et al recently used phage display to generate a
specific VCAM-1 ligand that is internalized allowing progressive concentration by endothelial cells. By tagging this ligand with iron it was possible to image VCAM-1 expression in mouse atherosclerosis.29 This is an ingenious approach, but, in comparison to MPIO, also has significant disadvantages. Firstly, cellular uptake is required, with potential for toxicity and ultimately loss of molecular specificity; secondly it was necessary to wait 48 hours after administration before imaging and thirdly, the mode of cellular uptake is so specific to VCAM-1 that the technology is not adaptable to other endovascular molecular targets.

The MPIO reported here are commercially available and can readily be substituted with other ligands of interest. We are currently exploring endogenous receptor ligands and novel peptides as mediators of MPIO binding. Technologies such as phage display can also facilitate identification of novel targets.29 We have purposely used relatively large MPIO (4.5 μm) because of their superiority as contrast vehicles. However, the conjugation technique can also be applied to smaller MPIO. The potential of MPIO for in vivo molecular imaging should also be emphasized. In this respect, Shapiro et al show excellent contrast using 1.63 μm molecular imaging of iron occurs at field strength of ≈2.1 T and would not be expected to increase at the higher field strengths used here.31 For molecular imaging, the contrast effect should increase as the radius of the particle increases, to the third power. The contrast effects observed by us and others are in the region of 100 to 200 μm per particle. Although the case is not proven here, this is of an order that may be detectable clinically.

Conclusions

We have shown that dual-ligand MPIO can be used for functional MRI of endothelial adhesion molecules in mouse atherosclerosis. We have exploited the opportunity of endovascular accessibility to use MPIO of similar size to circulating red blood cells (orders of magnitude larger than USPIO) that convey substantial contrast payload. To optimize MPIO binding under flow conditions in vivo, we have learnt from studies of leukocyte adhesion and computed models of dual-receptor binding to develop MPIO that target both P-selectin and VCAM-1. This work contributes an additional approach to the goal of disease characterization using functional molecular MRI probes.

Acknowledgments

The authors gratefully acknowledge the contributions of their colleagues; Phil Townsend, Lynn Clee, and Carol Williams. Dr Colin Clelland is thanked for contributing expertise in histology.

Sources of Funding

This work was funded by The Wellcome Trust (Intermediate Clinical Fellowship to R.C.).

Disclosures

Dr Choudhury is a named contributor on a patent application for the development of biodegradable imaging particles.

References


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Arterioscler Thromb Vasc Biol. 2008;28:77-83; originally published online October 25, 2007; doi: 10.1161/ATVBAHA.107.145466

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplementary Methods

**MPIO uptake by organs following *in vivo* administration**

To determine tolerance of *in vivo* MPIO injection and MPIO uptake in organs, anaesthetised apoE−/− mice (n = 8) were intravenously injected *in vivo* via the jugular vein with antibody-conjugated MPIO (6 x 10⁷ MPIO) as described above. MPIO were allowed to circulate for 30 min (n = 4 mice) or 24 h (n = 4). At the end of each time period, mice were terminally anesthetized, perfusion fixed with 4% PFA via the left ventricle, and lungs, spleen, liver and kidneys removed. Organs were paraffin-embedded and 15 µm sections were stained with haematoxylin and eosin. MPIO were quantified in 4-5 fields of view per section (3 sections per organ per mouse) using ImagePro Plus.

**Histology**

Aortic root specimens were dehydrated through graded ethanol solutions and Neo-clear (VMR, UK), paraffin-embedded and serially sectioned (15 µm thick). Rehydrated sections were stained with Masson’s Trichrome (VWR, UK). MPIO binding to the luminal side of atherosclerotic plaques was quantified in 3-4 histological sections per mouse by light microscopy.
Competitive inhibition of VCAM-MPIO binding in vitro

VCAM-MPIO (1.2x10^6) were pre-incubated for 1 h at RT with 5X soluble chimeric VCAM-1 mouse protein (Fc–VCAM-1) or Fc–ICAM-1 (R & D Systems, UK). \(^{11}\) TNF-\(\alpha\) stimulated (50 ng / mL) cells were incubated in triplicate with Fc-blocked MPIO for 30 min at RT. Unbound MPIO were removed by extensive washing with PBS. MPIO binding was assessed in 4 fields of view per sample using an inverted microscope (Nikon TE 2000; x 20 objective) and quantified using ImagePro plus. Cells were immunostained with rat anti–mouse VCAM-1 antibody (5 \(\mu\)g per ml PBS) for 1 hr at RT and detected with Alexa Fluor 488-conjugated rabbit anti-rat secondary antibody (1:100; Invitrogen, UK). Cells were mounted with Vectashield mounting media containing DAPI nuclear stain and analyzed by confocal microscopy (LSM 510, Zeiss).

Assessment of in vitro macrophage and sEND-1 cell uptake of MPIO by MRI

C57BL/6 mice (8 – 12 weeks) were injected intraperitoneally with 1 mL BIOgel beads (Bio-Rad, UK) in PBS (2% w / v). Elicited peritoneal macrophages were harvested by lavage with PBS/5 mM EDTA on day 4. Macrophages were cultured in OptiMEM (Invitrogen, UK) supplemented with L-glutamine and penicillin/streptomycin plated (1 \(\times\) 10^6 per 35 mm well). After 2 h, cells were washed with PBS. In addition, sEND-1 cells (1 x 10^6) were cultured in DMEM as described above.

Triplicate wells of macrophages and sEND-1 cells were incubated with MPIO (1.2 x 10^6 / 2 mL media) for 30 min or 2 h at RT, with constant mixing. Unbound MPIO were removed by extensive washing with PBS. Cells were embedded in 2% agarose, imaged and MR images analysed as above.
Figure I. Competitive inhibition of anti-VCAM-1-MPIO binding to TNF-α (50 ng / mL) stimulated sEND-1 cells in vitro. (A) Pre-incubation of anti-VCAM-1-MPIO with soluble VCAM-1 protein (Fc-VCAM-1) inhibited MPIO binding almost entirely (P<0.001), while pre-incubation with Fc-ICAM-1 had no effect. Data are mean ± SD for 4 fields per sample (triplicate experiments). (B) Confocal microscopy confirmed cell surface expression of VCAM-1 (green fluorescence) in TNF-α stimulated cells. Fc-ICAM-1 had no effect on anti-VCAM-MPIO binding (autofluorescent green spheres) while Fc-VCAM-1 abolished anti-VCAM-1-MPIO retention, despite demonstrable cell surface VCAM-1 expression. Blue represents DAPI staining for cell nuclei.
Extended legends for print-published figures

Figure 3. (A) Histological examination of aortic roots following left ventricular injection of single versus dual-targeted MPIO in terminally anesthetized apoE\(^{-/-}\) mice with beating hearts. (A) Three to four aortic root sections per mouse (15 \(\mu\)m thick) were examined for MPIO binding by light microscopy (x 40 magnification). There was minimal non-specific retention of control IgG-1-MPIO (n = 3 mice). Anti-VCAM-1-MPIO (n = 7 mice) and anti-P-selectin MPIO (n = 4 mice) showed similar levels of binding to aortic root plaque endothelium. However, dual-ligand MPIO (n = 6 mice) targeting VCAM-1 and P-selectin showed synergistically enhanced binding compared to anti-P-selectin MPIO or anti-VCAM-1-MPIO, respectively. (B) Light microscopy image (Masson trichrome) depicting dense dual-targeted MPIO binding to endothelium overlying atherosclerotic plaque in the aortic root (x 40 magnification). The MPIO are clearly distinguished as bright yellow spheres confined to the luminal surface of the vessel. The dark red bodies of irregular shape within the plaque are cell nuclei. Scale bar 20 \(\mu\)m. (*** P < 0.001; ** P < 0.01 and * P < .05).

Figure 4. High resolution \textit{ex vivo} MRI (11.7 T; resolution 25 x 25 x 25 \(\mu\)m) of aortic roots of apoE\(^{-/-}\) mice, 30 min after \textit{i.v.} injection of dual-targeted MPIO or IgG-1-MPIO (A) The number of bound dual-targeted MPIO, quantified by MRI, was 3.5-fold greater than IgG-1 MPIO (P < 0.01; n = 4 mice per group). (B) Dual-targeted MPIO were identified on MR images as distinct circular low signal areas adherent to endothelium overlying atherosclerotic plaque. A ‘halo effect’ was observed for MPIO in some
images, reflecting a partial volume effect: As individual MPIO were tracked through adjacent images, a dense low signal area was always present (top panel shows 3 consecutive MR images. Red arrows indicate bound MPIO, black arrows show MPIO passing in and out of the imaging plane). Scale bar 500 µm. (C) 3-D reconstruction of segmented images. Orthogonal slices were taken horizontally through the aortic valve and vertically along the axis of the aorta (panel i). Note the bloom effect of MPIO contrast in lesion areas of the aortic root and the absence of binding in atherosclerosis-free areas of ascending aorta. In panel (ii) the vertical slice was removed to illustrate the volume of MPIO contrast in the aortic root.