MRI-invisible pathology in murine cerebral malaria revealed by a novel contrast agent recognising activated platelet glycoprotein IIb/IIIa receptors

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Introduction:

Currently used MRI techniques in neurological disease are often limited in two important respects: (1) they reflect downstream injury, owing to relatively advanced pathology, and (2) while providing an indication of *severity*, they can not assess disease *activity*. Urgently needed are methods that accelerate diagnosis, quantify disease activity and guide specific therapy. Molecular imaging has the potential to realize this goal[1]. We have previously demonstrated the potential of microparticles of iron oxide (MPIO) conjugated to targeting ligands for the *in vivo* detection of cerebrovascular inflammation in rodent brain[2,3]. We now report the application of a similar MPIO-based agent that selectively targets activated platelets using an antibody that recognizes ligand-induced binding sites (LIBS) of GPIIb/IIIa, which are exposed only upon activation through receptor-ligand binding. Platelet sequestration in the cerebral microvasculature plays a pivotal role in the pathogenesis of cerebral malaria (CM). However, the mechanisms of platelet aggregation and adhesion are not completely understood. A number of proinflammatory cytokines are expressed within the brain parenchyma of mice and humans with CM, but it is not known whether platelet adhesion to the brain microvasculature is dependent on specific cytokine expression. In this study we have used the LIBS-MPIO agent to identify platelet accumulation in the cerebral microvasculature of mice with CM *in vivo*, and to determine the role of different cytokines (tumour necrosis factor [TNF] or interleukin-1β [IL-1β]) in this process.

Methods:

Targeted MPIO Synthesis: Autofluorescent cobalt-functionalised MPIOs (1µm diameter; 26% Fe) were conjugated to the histidine-tag of either the LIBS single-chain antibody or an equivalent irrelevant control antibody.
Animal Preparation: (i) **CM Model**: Female C57BL6-mice were injected intraperitoneally with 10^6 P. berghei ANKA-pRBC. At day 6 after inoculation animals were injected with either the LIBS-MPIO agent or control-MPIO (4 x 10^8 beads; 4.5 mg iron /kg body weight; n = 3 per group). (ii) **CNS Cytokine Injections**: 8-week-old male NMRI mice were microinjected stereotaxically in the left striatum with (a) 1µg of TNF in 0.5µl saline, or (ii) 1ng of IL-1β in 0.5µl saline, or (iii) 0.5µl saline alone. Animals were injected with either the LIBS-MPIO agent or control-MPIO (as above) at 5h, 12h or 24h after TNF injection, and at 12h after IL-1β injection.

MRI: MRI was performed in a 7T horizontal-bore magnet. T_2*-W 3D gradient-echo datasets were acquired as follows. **CM animals**: flip angle 35º, TR=15ms, TE=7ms, field of view 22.5x11.2x31.6mm, matrix size 192×96×360, 6 averages, acq. time ~30min. **Cytokine injected animals**: flip angle 35º, TR=50ms, TE=5ms, field of view 22.5x22.5x31.6mm, matrix size 192×192×360, 2 averages, acq. time ~1h. In each case the mid-point of the acquisition was 1.5-2.0h after MPIO injection. Data were zero-filled to 256x256x360; final isotropic resolution = 88µm^3. The brains were masked and low signal areas segmented and quantified.
Immunocytochemistry: Platelets were detected using rat anti-mouse glycoprotein IIb (CD41) polyclonal antibody and the number of platelet-positive elements quantitated. Cresyl-violet-stained brain sections were examined for the presence of MPIO by light microscopy.

Results:

CM animals: Using conventional MRI no abnormalities are detectable in CM mice at day 6 after inoculation. However, following LIBS-MPIO injection, negative MRI contrast was evident in and around cortical vessels on T_2*-W images, delineating areas of LIBS-MPIO binding (Fig. 1A; arrows). Conversely, CM-infected animals injected with control-MPIO exhibited no negative contrast (Fig. 1B). 3D reconstruction of the original MRI data stack, clearly shows binding of LIBS-MPIO in cortical regions of the brain (Fig. 1C), whilst injection of control-MPIO does not give rise to specific binding (Fig. 1D). Quantitation demonstrated a significantly greater volume of signal voids in the LIBS-MPIO-injected animals compared to control-MPIO injected animals (P=0.035). Immunohistochemically LIBS-MPIO binding was seen in areas of endovascular platelet aggregation.
Cytokine-injected animals: Following injection of LIBS-MPIO, areas of reduced signal intensity were observed on T_2*-W images from TNF injected animals, which appeared to delineate blood vessels (Fig 2A). In contrast, IL-1β injected mice given LIBS-MPIO showed no contrast effect (Fig. 2B), nor did animals injected with saline i.c. + LIBS-MPIO i.v. (Fig. 3C), or with TNF i.c. + control-MPIO i.v. (Fig. 3D). Quantitation demonstrated a significantly greater volume of signal voids in the TNF/LIBS-MPIO animals than any other group (P=0.01). Immunohistochemically, both MPIO binding and platelet adherence was detected in TNF injected animals, but not those injected with IL-1β. Both the volume of negative signal detected by MRI and the number of platelet positive elements determined immunohistochemically was maximal 12h post-TNF injection.

Fig. 1

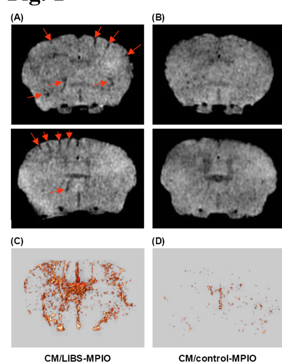
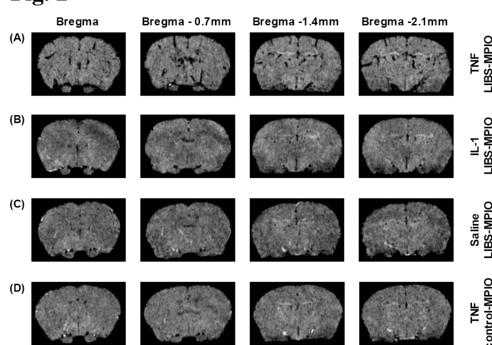


Fig. 2



Discussion:

This study provides proof of concept that in experimental models of human neurological diseases, targeted contrast agents can be used to detect pathology earlier than conventional, clinically used, MRI approaches or clinical assessment. Wall-adherent platelets were detected non-invasively *in vivo* in a model of cerebral malaria, at a time when conventional MRI techniques failed to reveal the presence of CNS pathology and before the appearance of overt clinical signs. Owing to the high specificity and sensitivity of our contrast agent, we were able to identify TNF as the mediator responsible for platelet aggregation. These results highlight the potential of targeted contrast agents for diagnostic, mechanistic and therapeutic studies.

References: 1. Sibson *et al* (2004) *Magn Res Med* 51:248-252
2. McAteer *et al.* (2007) *Nat Med* 13:1253-8
3. van Kasteren *et al*. (2006) *ISMRM Proceedings* 3508
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