MR Imaging of Tumor Associated Macrophages with Clinically-Applicable Iron Oxide Nanoparticles

Heike E. Daldrup-Link, MD
Daniel Golovko, MD
Brian Ruffell, PhD
David G. DeNardo, PhD
Rosalinda Castaneda, BA
Celina Ansari, MD
Jianghong Rao, PhD
Grigory A. Tikhomirov
Mike Wendland, PhD
Claire Corot, PhD
Lisa M. Coussens, PhD

1 Department of Radiology, Molecular Imaging Program at Stanford, Stanford University
2 Department of Medicine, University of Massachusetts Medical School, Worcester
3 Department of Pathology, Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, 513 Parnassus Ave, HSW450C, San Francisco, CA 94143
4 Department of Radiology and Biomedical Imaging, University of California San Francisco
5 Guerbet Group, Paris, France

Corresponding address: Heike E. Daldrup-Link, Molecular Imaging Program at Stanford, Stanford University, 725 Welch Road, Stanford, CA 94304, email: heiked@stanford.edu

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TRANSLATIONAL POTENTIAL
The presence of tumor-associated macrophages (TAMs) in adenocarcinomas correlates strongly with poor outcome in patients with breast cancer. Our data indicate that the FDA-approved iron oxide nanoparticle compound ferumoxytol (Feraheme) is preferentially phagocytosed by TAMs, but not by neoplastic tumor cells. In vivo, ferumoxytol administration was associated with an initial tumor perfusion, followed by tumor retention and persistent MR-enhancement at 24 hours after intravenous administration, which correlated with phagocytosed nanoparticles in TAMs. Together, these data indicate that ferumoxytol-enhancement may serve as a new biomarker for long-term prognosis and related treatment decisions that will support ongoing development of new immune-targeted therapies. Since ferumoxytol is FDA-approved as an iron supplement, this application is immediately clinically applicable as an imaging approach via an “off label” use.

ABSTRACT
Purpose: The presence of tumor-associated macrophages (TAMs) in breast cancer correlates strongly with poor outcome. The purpose of this study was to develop a clinically applicable, non-invasive diagnostic assay for selective targeting and visualization of TAMs in breast cancer, based on magnetic resonance (MR) imaging and clinically applicable iron oxide nanoparticles.

Experimental Design: F4/80-negative mammary carcinoma cells and F4/80-positive TAMs were incubated with iron oxide nanoparticles and were compared regarding MR signal changes and iron uptake. MMTV-PyMT transgenic mice harboring mammary carcinomas underwent nanoparticle-enhanced MR up to 1 hour (h) and at 24 h post injection (p.i.). The tumor enhancement on MR images was correlated with the presence and location of TAMs and nanoparticles on confocal microscopy.

Results: In vitro studies revealed that iron oxide nanoparticles are preferentially phagocytosed by TAMs, but not by malignant tumor cells. In vivo, all tumors demonstrated an initial contrast agent perfusion on immediate postcontrast MR images with gradual transendothelial leakage into the tumor interstitium. At 24 h p.i., all tumors demonstrated a persistent signal decline on MR scans. TAM-depletion via αCSF1 mAb lead to significant inhibition of tumor nanoparticle enhancement. Detection of iron using DAB-enhanced Prussian Blue staining, and immunodetection of CD68 localized iron oxide nanoparticles to TAMs, indicating that the MR signal effects on delayed MR images were largely due to TAM-mediated uptake of contrast agent.

Conclusion: These data indicate that tumor-enhancement with clinically applicable iron oxide nanoparticles may serve as a new biomarker for long-term prognosis, related treatment decisions and the evaluation of new immune-targeted therapies.
INTRODUCTION
While breast cancer (BC) has not historically been linked to underlying inflammation or infection, it exhibits tumor-associated inflammation marked by infiltration of leukocytes into developing tumors where increases in some leukocyte subsets parallels disease progression. In the majority of cases however, the natural immunity to cancer that is present is not protective, but instead fosters progression. Studies in transgenic mouse models of mammary carcinogenesis revealed that tumor-associated macrophages (TAMs) promote tumor growth and enhance pulmonary metastasis by high-level expression of epidermal growth factor (EGF) and activation of EGF-regulated signaling in mammary epithelial cells (MECs) critical for invasive tumor growth and metastatic dissemination. Histopathologic and flow cytometric evaluations have revealed that TAMs are the most abundant innate immune cell present in murine mammary carcinomas and in human breast cancers. TAM presence in several types of human cancer including breast, correlates with increased vascular density and worse clinical outcome. A clinically reliable non-invasive ex vivo imaging test, that could reliably detect and quantify TAMs, could be employed as a novel, widely applicable prognostic assay for stratifying individual patients to more aggressive and/or TAM-targeted therapies.

Intravenously injected superparamagnetic iron oxide nanoparticles (SPIO) are effective contrast agents for magnetic resonance (MR) imaging, that are phagocytosed by macrophages in various target tissues depending on their particle size and composition: Relatively large SPIO with hydrodynamic diameters in the order of 80-150 nm are rapidly phagocytosed by macrophages of the reticulo-endothelial system, such as liver, spleen and bone marrow while ultra small SPIO (USPIO) with diameters of less than 50 nm escape RES phagocytosis to some extend, leading to a prolonged blood pool circulation and accumulation and macrophage phagocytosis in inflamed tissues and tumors.

The goal of this study was to utilize novel USPIO to develop an immediately clinically applicable molecular imaging approach for enhanced imaging of TAMs in BC. Our imaging technique relies on the iron supplement ferumoxytol (Feraheme), recently FDA-approved for intravenous treatment of iron deficiency in patients. Ferumoxytol is also a USPIO compound, providing a strong signal effect on MR images and, thus, exerting properties of an MR contrast agent. Based on these properties, we postulated that ferumoxytol would be phagocytosed by TAMs in BC and thereby selectively enhance TAM presence on delayed, post-perfusion MR images with clinically applicable nanoparticles.

METHODS
Contrast Agents
Three ultrasmall superparamagnetic iron oxide nanoparticle compounds (USPIO) were investigated: 1) Ferumoxytol (Feraheme, AMAG Pharmaceuticals Inc.) is a USPIO nanoparticle applied for intravenous treatment of iron deficiency in patients with impaired renal function. Ferumoxytol consists of an iron oxide core and a carboxydextran coating. Ferumoxytol has a mean hydrodynamic diameter of 30 nm, an $r_1$ relaxivity of 38 s$^{-1}$mM$^{-1}$ and an $r_2$ relaxivity of 83 s$^{-1}$mM$^{-1}$ at 40 Mhz and at 37°C. Ferumoxytol was conjugated to FITC (Ferumoxytol-FITC) for detection by immunofluorescent microscopy. 2) P904 (Guerbet, Paris, France) is a USPIO compound currently in phase I clinical trials in Europe with plans for global distribution. P904 consists of an iron oxide core and a hydrophilic coating by a monomeric organic molecule with 20 hydroxylic groups. P904 has a mean hydrodynamic diameter of 21 nm, an $r_1$ of 14 s$^{-1}$mM$^{-1}$ and an $r_2$ relaxivity of 92 s$^{-1}$mM$^{-1}$ at 1 T and 37°C. 3) P1133 (Guerbet, Paris, France) is a pre-clinical USPIO with potential future clinical development. P1133 is based on P904, but also incorporates 8-10 folate moieties per nanoparticle in its coating, added via an amino PEG derivative of folic acid coupled on its g-carboxylic moiety to the carboxylate bearing iron core. P1133 has a mean hydrodynamic diameter of 26 nm, an $r_1$ relaxivity of 12 s$^{-1}$mM$^{-1}$ and an $r_2$ relaxivity of 95 s$^{-1}$mM$^{-1}$ at 1.5 T and 37°C.

Animal Model
This study was approved by the animal care and use committee at our institutions. MMTV-PyMT mice that spontaneously develop multifocal, multiclonal mammary adenocarcinomas were used at 12 to 14 weeks of life. Seven animals each received intravenous injections of ferumoxytol, P904, P1133. Six additional animals received injections of P1133 + free folic acid. Animal age, weight and tumor size were not significantly different between experimental groups that received different contrast agents ($p>0.05$). Additional experiments were carried out in seven postpubertal female FVB/n mice (10-12 weeks), which received injections of 50,000 PyMT-derived tumor cells into the right lower mammary fat pad for induction of orthotopic tumors. Three of these animals were treated with anti-CSF1 monoclonal antibody, clone 5A1 purified by the UCSF Hybridoma core using hybridoma obtained from the ATCC (#CRL-2702). The animals received an intraperitoneal injection of 2mg of anti-CSF1 mAb, including a 1mg starting dose, followed by a 0.5mg chaser doses on day 5 and 8, and ferumoxytol-enhanced MR imaging on day 9. Three additional animals served as controls and received intraperitoneal injections of PBS at the corresponding time points above, followed by ferumoxytol-enhanced MR imaging. One mouse received three subsequent MR studies on time point 0, 1h and 24 h without any contrast agent injection to confirm, that tumors do not show any changes in MR.
signal over a two day observation period. For all animals, MR imaging experiments were carried out when mammary tumors reached a size of approximately 1.0 cm.

**Macrophage Isolation and In Vitro Labeling**

Tumors from MMTV-PyMT mice at day 90-95, or PyMT-orthotopic tumors were isolated and digested in collagenase and DNAse (Roche Applied Sciences, Indianapolis, IN), strained over a cell strainer (BD Falcon, BD Biosciences, San Jose, CA) and incubated with phycoerythrin (PE) conjugated rat anti mouse F4/80 antibody (clone CI:A3-1, Caltag, Burlingame, CA). Cells were then incubated with anti PE magnetic beads and isolated over a magnetic column to provide F4/80 positive cells (macrophages and monocytes) and F4/80 negative cell fractions (malignant mammary epithelial cells and other stromal populations). In a previous study, we reported that F4/80+ cells represent Ly6G−Ly6C−CD11b+F4/80+ TAMs. 4 x 10⁶ of both F4/80-positive and negative cells were plated onto cell culture dishes in DMEM supplemented with 10% FCS. 200 μg [Fe]/ml of either Ferumoxytol, P1133 or P904 were added to the cell culture medium. Additional samples were incubated with P1133 + 1.67 μmol/ml of free folic acid, a dose that corresponds to 10 times the dose of folic acid engrafted onto P1133. Cells were incubated overnight at standard cell culture conditions (37°C, 5% CO₂). The next day, non-adherent cells were discarded and adherent cells were removed via a cell lifter. Removed cells were washed three times in PBS and resuspended in 400 μl of ficoll at a density of 1.07 g/ml and placed into 2.0 ml conical tubes for imaging. Experiments were performed in duplicates.

**In Vitro Cell Imaging and Data Analysis**

For *in vitro* imaging of nanoparticle-loaded cells, a clinical 3T scanner was used (Signa Excite HD, GE Medical Systems, Milwaukee, WI) with a standard wrist coil (USA Instruments, Aurora, OH). Test tubes were immersed in a water bath and a multiecho spin echo sequence was obtained with the following parameters: TE 15, 30, 45, 60 ms, TR 2000 ms, FOV 8x8 cm, matrix 256x196 pixels, slice thickness 2 mm and two acquisitions. Image processing was performed using MRVision software (MR Vision Co, Winchester, MA). T2 relaxation times were calculated assuming a monoexponential signal decay and using non linear least square curve fitting on a pixel by pixel bases.

**Determination of Cell Iron Content**

After imaging, cell samples were digested overnight in trypsin and placed in 10% HNO₃. Inductively coupled plasma optical emission spectroscopy (ICP-OES) was performed to quantify the iron content per sample (Perkin-Elmer, Waltham, MA).
In Vivo Imaging

Animals were randomly assigned to MR imaging when their tumor reached a size of approximately 1.0 cm. Imaging of MMTV-PymT mice before and after injection of different nanoparticles was performed with a 2 T Omega CSI-II MR scanner (Bruker Instruments, Fremont, CA) and imaging of mice before and after anti-CSF1-mAb treatment was performed with a 1 T desktop MR scanner (Aspect M2™ Compact High Performance MR System, Toronto, ON). Animals were anesthetized with isofluorane and placed on a recirculating water warming pad in a dedicated radiofrequency coil for high resolution MR imaging. A butterfly cannula filled with heparinized saline solution was introduced into the tail vein and left in place. T1, T2 and T2* weighted imaging sequences were obtained with the following parameters: T1 Spinecho (SE): TR 500 ms, TE 12 ms; T2 SE: TR 2000-2500 ms, TE 15, 30, 45, 60 ms (2T) and TE 20, 40, 60, 80 ms (1T); T2* Gradient echo (GE): TR 240 ms, TE 10 ms, flip angle 30 degrees (2T). MR scans were obtained with a field of view (FOV) of 3x3 cm (2T) or 6x6 cm (1T), a matrix of 128x128 or 200x200 pixels and a slice thickness of 1-2 mm.

Following precontrast T1, T2 and T2* weighted imaging, 24 PyMT animals received intravenous injections of 0.5 mmol [Fe]/kg ferumoxytol (n=7), P904 (n=7), P1133 (n=7), P1133 + 2.35 mmol/kg free folic acid (=100 times the dose of folate engrafted onto P1133; n=3) or P1133 + 0.235 mmol/kg free folic acid (=10 times the dose of folate engrafted onto P1133; n=3). Additional tumor-bearing mice after anti-CSF1-mAb treatment (n=3) or controls (n=3) were injected with 0.5 mmol [Fe]/kg ferumoxytol. After contrast media injection, without repositioning the mouse, 6 subsequent multiecho T2 SE sequences were obtained over the course of an hour, followed by T1- and T2*-weighted images. Mice were removed from the scanner, allowed to wake up and imaged 24 hours later with T1, T2 and T2* weighted sequences. T2-relaxation times of the tumor were calculated based on multiecho SE sequences and converted to R2-relaxation rates (R2=1/T2), which is proportional to contrast agent concentration. The relative change in R2 data between pre- and postcontrast MR scans, ΔR2 (%) was determined as a quantitative measure of tumor contrast enhancement.

Histology

After the last MR scan, at 24 hours post contrast media injection, mice were sacrificed, mammary tumors explanted, and placed in Optimal Cutting Temperature (OCT) compound on dry ice for histological processing. Samples were cut onto slides and warmed to room temperature, followed by fixation in 100% ice-cold acetone. Samples were then washed in H2O, and iron deposits in the tissue were detected using the Accustain Iron Stain Kit (Sigma-Aldrich, St. Louis, MO) according to the manufacture’s instructions, followed by signal enhancement with Fast 3,3 diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA) for 2 min. After
blocking sections with PBS containing 5% goat serum and 2.5% BSA (blocking buffer), sections were incubated overnight at 4°C with 0.5x blocking buffer containing either rabbit anti-mouse folate receptor α (1:100, Abcam, Cambridge, MA) or rat anti mouse folate receptor β (1:8, kind gift from Prof. Matsuyama, Kagoshima University, Japan) 29. Staining for folate receptor β was enhanced using a biotinylated anti-rat secondary antibody (1:200, Vector Laboratories, Burlingame, CA) and the Tyramide Signal Amplification kit (PerkinElmer, Waltham, MA). After extensive washing, sections were incubated overnight with FITC-conjugated rat anti-mouse CD68 (1:50, Serotec, Raleigh, NC), followed by Alexa 488 conjugated goat anti-FITC and either Alexa 546 conjugated donkey anti-rabbit or Alexa 546 conjugated Streptavidin (1:500 Invitrogen, Carlsbad, CA). For detection of Ferumoxytol-FITC, sections were stained with rat anti-mouse CD68 (1:100, Serotec), washed, and then incubated with a combination Alexa 546 conjugated donkey anti-rat and Alexa 488 conjugated goat anti-FITC. All slides were mounted using ProLong Gold with DAPI (Invitrogen) and analyzed using an LSM510 confocal microscope (Zeiss, Thornwood, NY).

Statistics
Statistical analysis comparing the differences of relaxation rates between mice receiving different contrast agents was performed with a Wilcoxon rank sum test. A t-test was used to determine the significance of differences between different cell samples as well as differences between age and tumor size of mice. A p value of less than 0.05 was considered significant.

RESULTS
F4/80-positive TAMs phagocytose USPIO in vitro
Following incubation with the iron oxide nanoparticle ferumoxytol, F4/80+ TAMs demonstrated a markedly decreased signal on T2-weighted MR images, while F4/80- cells demonstrated minimal signal changes compared to untreated controls (Figure 1A). Calculation of changes in relaxation rates (ΔR) as quantitative measures of the MR signal enhancement corroborated the qualitative findings with significantly higher ΔR2 data for ferumoxytol-exposed F4/80+ TAMs compared to ferumoxytol-exposed F4/80- cells consisting primarily of carcinoma cells (p>0.05). Determination of iron content in the samples revealed that increased iron uptake was responsible for the observed relaxation rate changes (Figure 1B).

Since both TAMs and malignant epithelial cells highly express the folate receptor, folate-linked USPIO have been recently developed for “tumor-targeted imaging” 24,30,31. F4/80+ TAMs incubated with folate-engrafted P1133 nanoparticles demonstrated significantly stronger visual and quantitative MR signal enhancement as compared to ferumoxytol and P904 (Figure 1). However, folate-engraftment also lead to significantly increased nanoparticle uptake and MR
enhancement of F4/80\(^+\) populations. The P1133-induced signal effect was inhibited by co-incubation with free folic acid to P904 levels (Figure 1), thus indicating that folate-targeting mediates increased USPIO uptake into mammary carcinomas.

**Ferumoxytol leads to persistent tumor enhancement on delayed, post-perfusion MR images, and corresponds to specific nanoparticle retention in TAMs**

We investigated 90-day-old MMTV-PyMT mice bearing late-stage mammary adenocarcinomas before and after intravenous injection of ferumoxytol, as well as syngeneic mice with PyMT-derived orthotopic mammary tumors. All tumors demonstrated an initial negative (dark) enhancement on immediate postcontrast T2-weighted MR images, that was most pronounced in the tumor periphery and which increased slowly and gradually up to 1.0-hour (h) post injection (p.i.). This corresponds to an initial blood pool perfusion of USPIO with slow, gradual transendothelial leakage of the nanoparticles into the tumor interstitium \(32-35\). At 24 h p.i. of ferumoxytol, all tumors demonstrated a persistent signal decline, which was most pronounced in tumor centers (Figure 2). Detection of iron using DAB-enhanced Prussian Blue staining, and immunodetection of CD68\(^+\) TAMs in tissue sections of mammary tumors localized ferumoxytol to CD68\(^+\) TAMs (Figure 3A). As it was difficult to show selective uptake using DAB-generated contrast due to high background, we also generated ferumoxytol-FITC to show colocalization by immunofluorescence using an Alexa 488 conjugated anti-FITC antibody. As shown in Figure 3B, ferumoxytol was specifically found within CD68\(^+\) TAMs, but not keratin 18-expressing malignant epithelial cells. While ferumoxytol was not found within all TAMs, these results indicate that the MR signal effects on delayed MR images were largely due to TAM-mediated uptake of contrast agent.

**USPIO-mediated TAM enhancement on delayed MR images can be increased by folate receptor-targeting of nanoparticles**

To determine if folate receptor targeting could enhance the MR images, we obtained additional MR images of MMTV-PyMT mice with late-stage mammary adenocarcinomas injected with the folate-engrafted USPIO P1133, the non-targeted analogue P904, or P1133 plus free folic acid. P1133 and P904 caused a nonspecific tumor-perfusion effect on T2-weighted images during the first hour post injection, which was not significantly different as compared to tumor-bearing mice injected with ferumoxytol (Figure 2). Delayed MR scans demonstrated a significantly stronger persistent tumor signal decline at 24 h p.i. of P1133 compared to ferumoxytol (p<0.05).

In vivo inhibition experiments with free folic acid are limited due to rapid liver uptake and renal elimination of free folic acid \(36\). Inhibition experiments with free folic acid at a 10 times increased dose as compared to the folate dose delivered with P1133 resulted in a minor, albeit
not significant inhibition of the P1133-induced tumor enhancement (p>0.05). Inhibition experiments with higher folic acid doses proved toxic in tumor-bearing mice, similar to previous reports. However, delayed MR scans demonstrated significantly less tumor enhancement at 24 h p.i. of folate-free P904 compared to folate-linked P1133 (p<0.05) (Figure 2). Since P1133 and P904 are chemically identical except for folate-engraftment on P1133, this data indicates increased nanoparticle uptake via folate receptor targeting.

We next evaluated TAMs versus epithelial cells for expression of α and β folate receptor in mammary tumors and revealed folate receptor α staining throughout epithelium, with no expression detectable on CD68+ TAMs (Figure 4A). In contrast, expression of folate receptor β was observed exclusively on CD68+ cells, although these represented only a portion of the total CD68+ TAMs found within tumors (Figure 4B) and appeared to be primarily associated with vascular and peripheral regions of mammary tumors. Consistent with the MR scans, Prussian Blue staining for iron was more prominent within tumors from mice injected with P1133 compared to P904 or ferumoxytol (Figure 4C). Furthermore, while some iron staining was observed in areas that did not appear occupied by TAMs (blue arrow), this was minor compared to staining within stromal areas likely enriched with TAMs (red arrow). Thus, while folate-engraftment did increase non-selective uptake of USPIOs by cells other than TAMs, these results indicate that folate modification of USPIOs may still improve their clinical use as evaluators of TAM presence within tumors.

Ferumoxytol-enhanced MRI detects TAM-depletion after anti-CSF1-mAb treatment

Imaging data of a control mouse that received three subsequent MR studies on time point 0, 1h and 24 h without any contrast agent injection confirmed, that the evaluated MMTV-PyMT tumors do not show any intrinsic changes in MR signal within a two-day observation period. Mice treated with anti-CSF1 monoclonal antibody demonstrated a similar ferumoxytol-tumor perfusion effect compared to untreated controls during the first hour after intravenous ferumoxytol-injection. However, at 24h p.i., anti-CSF1 mAb treated tumors showed less MR contrast effects and significantly smaller ΔR2 enhancement data compared to untreated controls (Figure 5). Corresponding confocal microscopy evaluations confirmed TAM-depletion of anti-CSF1 mAb treated tumors (Figure 5).

DISCUSSION

Results from this study demonstrate that ferumoxytol can be used as a reliable tool to quantitatively monitor macrophage presence in tumors, suggesting that this imaging technique can be readily applied as a surrogate measure to predict outcomes for patients with breast...
cancer, as well as to monitor TAM-targeted therapies now in clinical trials, and ultimately to monitor personalized and TAM-targeted therapies in clinical practice.

To the best of our knowledge, this is the first report of utilizing a clinically applicable nanoparticle for TAM-detection by MR imaging. Other investigators have reported TAM-enhancement with other nanoparticles, that are not clinically applicable, for either MR imaging or optical imaging. In addition, there have been reports of radiotracer-based approaches for TAM detection by positron emission tomography (PET). The latter is associated with radiation exposure and therefore not used routinely for breast imaging. MR imaging, on the other hand, is radiation-free, established for breast cancer detection and integrates near-microscopic anatomical resolution, high sensitivity and excellent soft tissue contrast. While histological methods for quantifying TAMs are more precise, they are invasive, limited to one or few observations and not representative of the whole tumor in the case of biopsies.

Preclinical and clinical evidence indicates that a chronic presence of diverse leukocyte subsets in stroma of breast cancers promotes tumor growth and metastasis. TAMs play a significant pro-tumorigenic role in this context by augmenting neoplastic cell survival and motility via elaboration of cytokines, chemokines, proteases and reactive oxygen species. In addition, while TAMs have been found to potentiate pulmonary metastasis of mammary adenocarcinomas by enhancing angiogenesis via regulating vascular endothelial growth factor (VEGF) bioavailability and supplying epidermal growth factor (EGF) to mammary epithelium, they also can suppress protective adaptive immune responses. Exuberant macrophage recruitment to BC has been reported to be strongly associated with poor prognosis, both in animal models and in patients. Although phagocytotic capacity can be altered by in vitro polarization of macrophages, we have no evidence that ferumoxytol uptake corresponds to a particular TAM phenotype. Regardless, aggressive human BCs have been reported to contain few (if any) T\(_{\text{H1}}\)-polarized macrophages, and in the MMTV-PyMT transgenic model in particular, TAMs are strongly T\(_{\text{H2}}\)-polarized by interleukin 4.

Ferumoxytol-enhancement is a new, non-invasive indicator for TAM-tumor infiltration, that may serve as a novel biomarker for BCs with poor outcome and which may be utilized to stratify TAM-positive tumors for immune-targeted therapies. Ferumoxytol-enhanced TAM-imaging could also be applied to examining effects of immune-response modifiers in BC progression by direct \textit{in vivo} imaging, that could in turn lead to development of new classes of anti-inflammatory and anti-neoplastic agents. There have been multiple approaches for specific targeting and/or blockade of TAMs for therapeutic purposes, some of which are currently in clinical trials based on experimental data showing that genetic, immunological or pharmacological blockade of colony stimulating factor (CSF)1 or its receptor (CSF1R) decreases TAM presence in tissues and in experimental solid tumors, correlating with...
diminished tumor angiogenesis, and reduced primary tumor growth and pulmonary metastasis \(^{49-53}\). Since these therapies are not cytotoxic, biomarkers of their efficiency at inducing macrophage depletion would be of great clinical benefit. Moreover, since clinical trials of new therapeutic drugs and new combination therapies are expensive and take years to complete, the immediate value and impact of imaging TAMs and/or TAM-depletion via MR imaging would be immense.

We recognize several limitations with this approach. Studies reported herein were performed with ferumoxytol doses of 0.5 mmol/kg. Previously described ferumoxytol doses in humans were in the order of 0.035 – 0.072 mmol/kg \(^{18,19}\). Iron oxide nanoparticles are generally applied in higher doses in rodents as opposed to humans in order to compensate for the relatively faster blood half life in rodents. However, future clinical applications must demonstrate if the currently applied dose in patients is sufficient for TAM-detection, or if the dose can be safely increased. Of note, ferumoxytol showed an excellent safety profile in more than 700 patients \(^{17}\). Larger superparamagnetic iron oxide nanoparticles (SPIOs, diameter > 50 nm) provide higher cellular uptake via ex vivo labeling. However, SPIO are rapidly phagocytosed by macrophages in liver, spleen and bone marrow and do not reach TAMs in vivo. USPIO on the other hand, are not as quickly recognized by the RES, have a longer circulation time and can therefore leak into tumor interstitium, where they can be phagocytosed by TAMs. Thus, for “in vivo TAM labeling”, USPIO are advantageous\(^{32,33}\).

As demonstrated by our data, an alternative approach to increase the sensitivity of MR imaging would be to utilize folate-engrafted nanoparticles. Although such particles are currently not available for clinical use, precursors of such compounds are currently entering clinical trials and thus, folate-engrafted derivatives may become clinically available in the future. Folate-engraftment enhances nanoparticle uptake via the folate receptor beta, which is highly expressed on TAMs \(^{31}\). Several investigators including us have reported uptake of USPIO and folate-engrafted USPIO by neoplastic cells, which may be a confounding variable when aiming for TAM detection \(^{24,54,55}\). However, data presented here reveal that the ferumoxytol and P1133 uptake in malignant epithelial cells is significantly lower as compared to macrophage uptake, leading to negligible interferences of our imaging approach.

In conclusion, we have demonstrated that iron oxide nanoparticle-enhanced MR imaging can be utilized to detect TAMs in a mouse model of mammary carcinogenesis. Ferumoxytol is a clinically available nanoparticle that can be readily applied for TAM imaging in patients with BC via an “off label” use. Macrophage enhancement can be increased by using folate-engrafted nanoparticles which may become available for clinical use in the near future. Clinical studies are under way to evaluate these finding in patients.
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REFERENCES:

FIGURE LEGENDS

Figure 1. In vitro MR scans of iron oxide nanoparticle-labeled cells with corresponding quantitative MR signal enhancement and spectrometry data. (A) Axial T2-weighted MR images through test tubes containing F4/80⁺ versus F4/80⁻ cells labeled overnight with Ferumoxytol, P904, P1133 alone or P1133 with free folic acid (FFA). Cells were kept in suspension in ficoll solution and test tubes were placed in a water bath to avoid artifacts by surrounding air (which would cause a dark MR signal). Image parameters: 3 Tesla, SE 2000/60 (TR/TE in ms). (B) Corresponding R2 relaxation rates, i.e. quantitative measures of the MR signal effect, of iron oxide nanoparticle labeled and unlabeled F4 F4/80⁺ versus F4/80⁻ cells, displayed as mean +/- standard deviation from duplicate experiments. (C) Iron content of the same cell samples as shown in B, as determined by mass spectrometry.

Figure 2. In vivo MR imaging of iron oxide nanoparticles. (A) T2-weighted SE images of representative mammary tumors in MMTV-PyMT mice prior to (precontrast), 1 hour (h) and 24 h after administration of 0.5 mmol [Fe] / kg of ferumoxytol, P904 or P1133. The iron oxide nanoparticle-based contrast agents cause a negative (dark) signal effect in the tumor tissue on these scans (arrows point to tumors). (B) Quantitation of MR signal enhancement (delta R2 measurement), of mammary tumors in MMTV-PyMT mice before and after iron oxide-nanoparticle administration, displayed as means +/- standard deviation (n=7 mice/group, except P1133+FFA which contained 3 mice). Note that all tumors show a nanoparticle retention at 24 hours, which is most pronounced for the folate-linked nanoparticle P1133.

Figure 3. Uptake of ferumoxytol by TAMs in vivo. (A) Localization within OCT-embedded mammary tumors of ferumoxytol (iron; black contrast) to CD68⁺ macrophages (green) using
phase contrast of DAB staining and confocal microscopy. (B) Localization of ferumoxytol-FITC (green) to CD68⁺ macrophages (red) but not Keratin 18⁺ carcinoma cells (red) within mammary tumors. Scale bars are shown in images.

**Figure 4. Folate receptor expression and folate-targeted uptake of nanoparticles.** (A) Staining for folate receptor α (FRα; red) and CD68⁺ macrophages (green) demonstrates that expression of FRα is localized to carcinoma cells within mammary tumors. (B) A subpopulation of CD68⁺ macrophages (green) express folate receptor β (FRβ; red staining). (C) Prussian Blue staining for iron with DAB enhancement within mammary tumors from mice injected with ferumoxytol, P904 or P1133. Scale bars are shown in images.

**Figure 5. Ferumoxytol-MRI detects TAM-depletion non-invasively in vivo**

(A) Quantitative MR signal enhancement (delta R2 measurement) of MMTV-PyMT mammary before and after iron oxide-nanoparticle administration, displayed as means +/- standard deviation of three mice treated with anti-CSF1 monoclonal antibody and three controls. An additional control mouse underwent serial MRI without any contrast agent injection in order to confirm, that the evaluated MMTV-PyMT tumors do not show any intrinsic changes in MR signal within a two-day observation period. Note that mice treated with anti-CSF1 mAb demonstrated significantly smaller ΔR2 enhancement data compared to untreated controls. (B) Corresponding confocal microscopy evaluations confirmed TAM-depletion of anti-CSF1 mAb treated tumors. The detected quantity of CD68⁺ macrophages was markedly higher in anti-CSF1 mAb treated tumors compared to untreated control tumors.
A. In vitro MR imaging

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<tr>
<td>F4/80-</td>
<td><img src="image2" alt="Image" /></td>
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</table>

B. In vitro MR signal

![Graph](image3)

R2 (1/s)

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<th>unlabeled ferumoxytol</th>
<th>P904</th>
<th>P1133</th>
<th>P1133 + FFA</th>
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<td>F4/80-</td>
<td><img src="image5" alt="Bar" /></td>
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</table>

C. Cellular iron content

![Graph](image6)

picogram Fe/cell

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<tbody>
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<td>F4/80-</td>
<td><img src="image8" alt="Bar" /></td>
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</table>
A. In vivo MR imaging

B. In vivo tumor enhancement
Daldrup-Link et al. Figure 3

A  Ferumoxytol  Non-injected

Iron DAPI Iron DAPI

B  Epithelial

DAPI Keratin 18  DAPI Keratin 18 Ferumoxytol

DAPI Ferumoxytol  DAPI CD68 Ferumoxytol

DAPI CD68  DAPI CD68 Ferumoxytol

Macrophage

Macrophage

DAPI CD68  DAPI CD68 Ferumoxytol

DAPI CD68  DAPI CD68 Ferumoxytol

5 μm 5 μm 5 μm

Downloaded from clincancerres.aacrjournals.org on April 12, 2017. © 2011 American Association for Cancer Research.
**Figure 5**

**A. Tumor MR Contrast Enhancement**

<table>
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<tr>
<th>Group</th>
<th>1 h.p.i.</th>
<th>24 h.p.i.</th>
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</thead>
<tbody>
<tr>
<td>Control (no contrast)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control (no mAb)</td>
<td>5</td>
<td>10</td>
</tr>
<tr>
<td>αCSF1 mAb treated</td>
<td>15</td>
<td>20</td>
</tr>
</tbody>
</table>

* indicates statistically significant difference.

**B. Control vs. αCSF1 mAb**

DAPI and CD68 staining images showing differences in tumor contrast.
Clinical Cancer Research

MR Imaging of Tumor Associated Macrophages with Clinically-Applicable Iron Oxide Nanoparticles

Heike E Daldrup-Link, Daniel Golovko, Brian Ruffel, et al.

Clin Cancer Res Published OnlineFirst July 26, 2011.

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