Citing this paper
Please note that where the full-text provided on King's Research Portal is the Author Accepted Manuscript or Post-Print version this may differ from the final Published version. If citing, it is advised that you check and use the publisher's definitive version for pagination, volume/issue, and date of publication details. And where the final published version is provided on the Research Portal, if citing you are again advised to check the publisher's website for any subsequent corrections.

General rights
Copyright and moral rights for the publications made accessible in the Research Portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognize and abide by the legal requirements associated with these rights.

• Users may download and print one copy of any publication from the Research Portal for the purpose of private study or research.
• You may not further distribute the material or use it for any profit-making activity or commercial gain
• You may freely distribute the URL identifying the publication in the Research Portal

Take down policy
If you believe that this document breaches copyright please contact librepure@kcl.ac.uk providing details, and we will remove access to the work immediately and investigate your claim.
Monitoring of In Vivo Function of Superparamagnetic Iron Oxide Labelled Murine Dendritic Cells during Anti-Tumour Vaccination

Richard Tavare1*, Pervinder Sagoo2,3, Gopal Varama1, Yakup Tanriver2, Alice Warely4, Sandra S. Diebold5, Richard Southworth1, Tobias Schaeffter1, Robert I. Lechler2, Reza Razavi1, Giovanna Lombardi2*, Gregory E. D. Mullen1*

1 Division of Imaging Sciences and Biomedical Engineering, Department of Imaging Chemistry and Biology, King’s College London, St. Thomas’ Hospital, London, United Kingdom, 2 MRC Centre for Transplantation, King’s College London, Guy’s Hospital, London, United Kingdom, 3 NIHR Biomedical Research Centre, Guy’s and St Thomas’ NHS Foundation Trust and King’s College London, London, United Kingdom, 4 Centre for Ultrastructural Imaging, King’s College London, Guy’s Campus, London, United Kingdom, 5 Division of Immunology, Infection and Inflammatory Disease, Peter Gorer Department of Immunology, King’s College London, Guy’s Hospital, London, United Kingdom

Abstract

Dendritic cells (DCs) generated in vitro to present tumour antigens have been injected in cancer patients to boost in vivo anti-tumour immune responses. This approach to cancer immunotherapy has had limited success. For anti-tumour therapy, delivery and subsequent migration of DCs to lymph nodes leading to effective stimulation of effector T cells is thought to be essential. The ability to non-invasively monitor the fate of adoptively transferred DCs in vivo using magnetic resonance imaging (MRI) is an important clinical tool to correlate their in vivo behavior with response to treatment. Previous reports of superparamagnetic iron oxides (SPIOs) labelling of different cell types, including DCs, have indicated varying detrimental effects on cell viability, migration, differentiation and immune function. Here we describe an optimised labelling procedure using a short incubation time and low concentration of clinically used SPIO Endorem to successfully track murine DC migration in vivo using MRI in a mouse tumour model. First, intracellular labelling of bone marrow derived DCs was monitored in vitro using electron microscopy and MRI relaxometry. Second, the in vitro characterisation of SPIO labelled DCs demonstrated that viability, phenotype and functions were comparable to unlabelled DCs. Third, ex vivo SPIO labelled DCs, when injected subcutaneously, allowed for the longitudinal monitoring by MR imaging of their migration in vivo. Fourth, the SPIO DCs induced the proliferation of adoptively transferred CD4+ T cells but, most importantly, they primed cytotoxic CD8+ T cell responses to protect against a B16-Ova tumour challenge. Finally, using anatomical information from the MR images, the immigration of DCs was confirmed by the increase in lymph node size post-DC injection. These results demonstrate that the SPIO labelling protocol developed in this study is not detrimental for DC function in vitro and in vivo has potential clinical application in monitoring therapeutic DCs in patients with cancer.


Editor: Francesco Dieli, University of Palermo, Italy

Received February 1, 2011; Accepted April 2, 2011; Published May 27, 2011

Copyright: © 2011 Tavare et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: The authors acknowledge the financial support from King’s College London, Division of Imaging Sciences, MRC Centre of Transplantation, The Department of Health via the National Institute for Health Research (NIHR) comprehensive Biomedical Research Centre award to Guy’s and St Thomas’ NHS Foundation Trust in partnership with King’s College London and King’s College Hospital NHS Foundation Trust and The British Heart Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: greg.mullen@kcl.ac.uk (GM); giovanna.lombardi@kcl.ac.uk (GL)

# Current address: Department of Molecular and Medicinal Pharmacology, Crump Institute for Molecular Imaging, California NanoSystems Institute, University of California Los Angeles, Los Angeles, California, United States of America

Introduction

The ability to non-invasively image adoptively transferred dendritic cell (DCs) in vivo during cellular immunotherapy could be used to assess the in vivo behavior of injected DCs and correlate it to the clinical response to therapy. In particular, the ability to quantify the number of injected DCs that have migrated from the injection site to the draining lymph nodes (LN) would be a valuable clinical tool. To date, however, there has been limited success of DC based immunotherapies and many questions remain with regard to appropriate sites of delivery, frequency of delivery, cell number, DC phenotype, and optimal antigen for the most robust immune stimulation or tolerance induction [1]. Molecular imaging may provide some of the answers to these questions by using superparamagnetic iron oxide (SPIO) labelled DCs (SPIO DCs). To date, studies of SPIO DCs in vivo have focused mostly on evaluating their migratory properties using MRI and evaluating cell surface markers that are required for afferent LN migration but not on SPIO DC in vivo function [2,3,4,5,6].

Initial proof of concept studies demonstrated that DCs could be labelled with SPIOs and then monitored in vivo over time by MRI [2]. The efficiency of SPIO labelling of DCs was further enhanced by using SPIOs conjugated to anti-CD11c antibodies, resulting in a 50-fold increase in SPIO labelling of DCs [2]. However, this intense loading with SPIOs, with 30 pg or higher of iron per cell, was shown to adversely affect DC viability and migration by
Verdijk et al. [3]. The use of MRI for the in vivo analysis of SPIO labelled DCs has also led to the discovery that the receptors CCR7 [4] and RAGE [5] are required for DC migration. Furthermore, MRI has allowed the study of DC migration to draining LNs in a semi-quantitative manner, showing a correlation between hypointensity in draining LNs and number of DCs injected [6]. In a recent study, rapamycin treated tolerogenic DCs were used in a graft-vs-host disease (GVHD) model where recipient tolerogenic DCs prolonged survival and decreased GVHD score on day seven post-bone marrow transplantation [7]. Monitoring of DC migration in this GVHD model, using tolerogenic DCs labelled with SPIO, showed hypointensity via MRI in the cervical LN eight days after injection, demonstrating the application of this approach to in vivo study of DC function. However, this study did not perform a comparison of SPIO labelled and unlabelled DCs. Similarly the cell numbers that migrated to LNs after injection were not quantified using SPIO labelled DCs [7]. Finally, in a clinical study the use of MRI and SPIO labelled human DCs has demonstrated that only around 50% of the cases the DCs were correctly injected into the lymph node despite ultrasound guidance and showed subsequent migration to other local LNs [8].

Altogether, investigations to date have not studied the effect of SPIO labelling on DC immunological function in vivo compared to their unlabelled counterparts. So far SPIO labelled and unlabelled DCs have been compared for their ability to initiate T cell response by proliferation assay and the ability of SPIO labelled and unlabelled DCs to induce T cell proliferation in vitro. Baumjohann et al. demonstrated that the capacity of SPIO DCs to prime an antigen-specific syngeneic T cell response by in vitro re-challenge was reduced by ~90% compared to unlabelled DCs [4]. The authors suggested that the antigen presentation capacity of SPIO DCs remained intact in vivo but that SPIO-loading may have minor influences on migration and uptake or processing of antigens [4]. In a more recent paper using a low SPIO labelling efficiency of ~6 pg iron per cell, a 15% decrease in ex vivo T cell proliferation was demonstrated when compared to unlabelled DCs [6]. The decreased stimulatory capacity of SPIO DCs compared to unlabelled DCs was confirmed further by demonstrating a decrease in their endocytic function by using dextran-FITC or fluorescently labelled apoptotic cells [6]. The explanation offered by the authors was that the dextran coat on SPIOs may impede the uptake of other glycosylated antigens through endocytosis. Utilising GFP+ DCs, they also showed that the number of GFP+ cells in the draining LN was reduced when DCs were SPIO labelled. The same group has repeated this using micrometer-sized iron oxides (MPIOs) and reported similar results where MPIO labelling decreased DC migration in vivo [9].

In this study, SPIO DC labelling efficiency, intracellular SPIO distribution, viability, phenotype, and ability to induce T cell proliferation were assessed in vitro and compared to unlabelled DCs. MRI was applied to monitor SPIO DC migration in vivo to the popliteal LN. When monitoring the migration of DCs over time, both SPIO labelled or unlabelled caused a similar increase in popliteal LN volume. DCs were labelled with sufficient SPIO to induce contrast hypointensity via MR imaging without affecting their in vivo function as compared to unlabelled DCs. This was demonstrated by an in vivo proliferation assay and the ability of SPIO DCs to induce protection against an Ova-B16 melanoma tumour challenge.

Materials and Methods

Ethics statement

Animal studies were carried out in accordance with UK Research Councils' and Medical Research Charities' guidelines on Responsibility in the Use of Animals in Bioscience Research, under a UK Home Office license (PPL# 70/6473; Title: Mechanisms of immunological response to foreign antigens).

Mice, culture media, reagents and antibodies

C57BL/6 (H2b) and CBA/Ca (H2k) mice were ordered from Harlan Olac (Bicester, UK). OT-II and DO11.10 were bred and maintained in the Biological Services Unit at King’s College London. RPMI 1640 medium (Sigma, Poole, UK) supplemented with 5 mM L-Glut (Invitrogen, Paisley, UK), 100 U/mL penicillin (Invitrogen), 100 μg/mL streptomycin (Invitrogen), 10% FCS (Harlan Sera-Lab, Loughborough, UK), 1 mM Hepes (Invitrogen) and 0.05 mM mercaptoethanol (Invitrogen) is used for all in vitro assays. DC media also contains 5% (vol/vol) supernatant from a granulocyte macrophage colony-stimulating factor (GM-CSF) secreting transfected cell line [10]. For T cell purification and washing steps, RPMI 1640 medium supplemented with 2% fetal calf serum (FCS) was used. Flow Cytometry was performed using a FACS Calibur (BD Biosciences) and the following phycoerythrin (PE)-conjugated antibodies (Abs): MHC-class I (Iκbα), MHC-class II (Iκbα), CD80, CD86, CD40, CD11c, CD54 and CCR7. All antibodies and isotype controls were used according to manufacturer’s instructions (BD Biosciences).

Generation and SPIO labelling of mature bone marrow derived dendritic cells

DCs where generated as previously described [10,11]. Briefly, bone marrow was isolated and treated with red blood cell (RBC) lysis Ack Buffer (150 mM NaCl, 1 mM KHCO3, 0.1 mM Na2-EDTA). Cells were then treated with a mixture of rat anti-mouse hybridoma supernatants containing anti-H2-2d/LA/Ab (M5/114.15.2; TIB-120; ATCC), anti-CD45R/B220 (RA3-3AI/6.1; ATCC), anti-CD4 (YTS191; Therapeutic Immunology Group, Oxford, UK) and anti-CD8 (YTS169; Therapeutic Immunology Group). After washing with PBS, cells were incubated with goat anti-rat IgG DynaBeads (Dyna, Oslo, Norway) before separation in a magnetic field for negative isolation. Cells are resuspended in complete media plus GM-CSF and plated on 24-well plates (Barloworld Scientific, Staffordshire, UK) in 1 mL fractions at 1–1.5×106 cells/mL. Complete media plus GM-CSF was changed on days 2 and 4, removing non-adherent cells. On Day 7 DCs were harvested, re-plated at 1×106 cells/mL in a 24-well plate and concurrently incubated with or without 100 μg/mL of SPIO. Cells were washed three times with PBS before use in subsequent assays. The clinically approved SPIO agent Endorem was purchased from Guibert, France.

Iron Quantification of SPIO labelled DCs

The average iron quantity per cell was calculated using the previously described calorimetric ferrozine-based assay [12]. Briefly, 2×105 cells were counted, pelleted and lysed at −80°C for 30 min. and then shaken at room temp for 2 h in 200 μL of 50 mM NaOH. 100 μL of lysate was added to 100 μL of 10 mM HCl and 100 μL of iron releasing agent (fresly mixed solution of equal volumes of 1.4 M HCl and 4.5% (w/v) KMnO4 (Merek) in dH2O) for 2 h at 60°C. Once cool, 30 μL of iron detection reagent was added (6.5 mM Ferrozine, 6.5 mM Neocuproine, 2.5 M ammonium acetate, 1 M ascorbic acid) for 30 min. 280 μL was transferred to a 96-well plate and absorbance is read at 550 nm. All samples were run in triplicate and compared to known concentrations of ferric chloride.
Electron microscopy

SPIO labelled and unlabelled DCs were fixed for 1 h in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and post-fixed in 1% aqueous osmium tetroxide. Samples were then dehydrated through a graded series of ethanol and embedded in TAAB resin. Sections 70 nm thick were cut using a Leica ultracut E ultra microtome (Leica, Milton Keynes, UK), mounted on 200 mesh Cu grids (Agar Scientific, Stanstead, UK), and stained with 1.5% uranyl acetate in 50% ethanol and 0.15% lead citrate before viewing on a Tecnai T12 electron microscope (FEI, the Netherlands). Images were captured using a Gatan BioScan 792 camera (Gatan, Abington Oxon, UK). Energy filtered transmission electron microscopy (EFTEM) is a transmission electron microscopy technique in which those electrons that have suffered specific energy loss due to interaction with the atomic nuclei of a given element are used to form an image of the distribution of that element. EFTEM was carried out using a G2 Sphera 200 kV electron microscope (FEI, the Netherlands) fitted with a LaB6 emitter. Sections were imaged in transmission mode. Maps of Fe distribution were obtained with a GIF 2002 imaging filter, using TIA software.

Magnetic Resonance Imaging

For the in vitro study MR relaxometry measurements were performed on a 1.5 T clinical whole-body MR unit (Achieva; Philips Medical Systems, UK). In order to study the influence of the labelled cells on the MRI contrast, different concentrations of SPIO labelled cells were imaged by multi-gradient-echo MR-sequence. For this, SPIO labelled DCs were diluted in 2-fold dilutions from 1×10⁶ to 6.25×10⁵ cells/mL gelatin (Sigma) and aliquoted in glass tubes that were then added to a gelatin filled dish. Also, free SPIO in gelatin was diluted in 2-fold dilutions from 50 to 0.78 μg SPIO per mL.

For quantification of the labelling process R₂ values were measured using a multi-spin-echo and gradient-echo sequences respectively to give magnitude based images at increasing echo-times (TEs). Taking the data on a pixel-by-pixel basis and plotting against its TE, a mono-exponential decay function of the form M₀exp(-R₂*TE) was fit to give maps of the respective relaxation rates. The R₂ map was used for positive contrast. For this, SPIO labelled DCs were diluted in 2-fold dilutions from 1×10⁶ to 6.25×10⁵ cells/mL gelatin (Sigma) and FlowJo (Oregon, USA). For phenotype surface staining, 5×10⁵ DCs were incubated according to manufacturer’s instructions with various antibodies in FACS Buffer (PBS plus 1% [vol/vol] FCS and 0.01% [w/vol] sodium azide) for 30 min. at 4°C in the dark followed by two washes in FACS Buffer. For in vivo proliferation experiments, flow cytometry is described in the methodology of the assay.

Dendritic cell viability assays

Viability assays were performed at 4 or 18 h of SPIO labelling at 50, 100, or 150 μg SPIO per mL culture media. DCs incubated with SPIO under various labelling conditions were harvested and counted after the labelling procedure in a haemocytometer in the presence of Trypan Blue (Sigma). For the MITT assay, DCs incubated with SPIO under various labelling conditions were harvested and re-plated in a flat-bottomed 96-well plate (Iwaki) at 1×10⁵ cells per 100 μL media in triplicate. 10 μL of 5 mg/mL 3-[4,5-dimethylthiasol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well and incubated at 37°C for 4 h. 100 μL of 10% SDS in 1 mM HCl was added to each well and incubated at 37°C overnight. The absorbance of each well was read in a BioTek spectrophotometer at 570 nm.

CD4⁺ T cell isolation

Spleens and lymph nodes (LN) were homogenized over 70 μm cell strainers (Beckman Coulter) to obtain a single cell suspension that was treated with ACK buffer. The washed cells were then incubated with a mixture of rat anti-mouse hybridoma supernatants containing anti-H2-Eb/A⁺/ident, anti-CD4/5R/B220, and anti-CD8 followed by goat anti-rat IgG DynaBeads before separation in a magnetic field. Purity of negatively selected CD4⁺ population was >93% before immediate use in further assays.

In vitro proliferation assays

All proliferation assays were done in triplicate in 96-well plates with a volume of 250 μL. For peptide pulse OT-II proliferation assays, C57BL/6 DCs that had been treated ± SPIO and with LPS were pulsed with Ova₃₂₂₋₃₃₂ that binds MHC-class II (A⁺) at 0.1 μg/mL for 30 min. After peptide pulse, the DCs were irradiated (~3000 rad) and plated at 5×10⁴ DCs/well. DCs were incubated with freshly isolated CD4⁺ T cells from OT-2 transgenic mice from the spleen and lymph nodes and were mixed at various DC:T cell concentrations. Proliferation was assessed by ³H-thymidine incorporation in the last 18 h of 4-day cultures. Mixed lymphocyte reactions (MLRs) were performed similarly with irradiated DCs incubated with freshly isolated CBA/Ca CD4⁺ T cells at various DC:T cell concentrations. Again, proliferation was assessed by ³H-thymidine incorporation in the last 18 h of 4-day cultures. For protein pulse assays, DCs were treated with 10 mg/mL Ova protein (Sigma) for 4 h before SPIO labelling. After irradiation, DCs were incubated with isolated CD4⁺ T cells from OT-II mice at various DC:T cell concentrations. Proliferation was assessed by ³H-thymidine incorporation in the last 18 h of 4-day cultures.

In vivo proliferation assay

Freshly isolated OT-II CD4⁺ T cells were incubated with 1 μM CFSE in 10 mL PBS per 1×10⁶ cells for 5 min. at 37°C and checked by flow cytometry for effective staining. 4×10⁶ cells in PBS were i.v. injected on Day 6. On Day 7 of DC culture, 1×10⁵ mature Ova₃₂₂₋₃₃₂ pulsed DCs labelled with or without SPIO were injected in PBS s.c. in the heel. Animals were culled on Day 11 and specific organs were harvested. 1.5×10⁶ cells isolated were stained with anti-CD4-APC and 1×10⁶ events were collected for analysis by flow cytometry. Percent original dividing
cells was the percentage of cells from the parent population that divided assuming that no cells died during the experiments. It was calculated by: (the total number of parent cell that had divided)/the calculated number of total parent cells)x100. The proliferation index (PI) was the average number of divisions that the dividing cells have undergone. It was calculated by dividing the total number of events by the calculated number of parent cells.

**Tumour vaccination**

On Day 0, C57BL/6 mice were s.c. immunised with $1 \times 10^6$ DCs pulsed with Ova257–264 (SIINFEKL), labelled with and without SPIOs. On Day 4, $1 \times 10^6$ B16 melanoma tumour cells expressing Ovalbumin and GFP were i.v. injected. Lungs were then harvested on Day 18 and counted for tumour nodules until a limit of 250 nodules per lung.

**Histology**

Live DCs were allowed to bind to glass coverslips (Hendley, Essex, UK), fixed with 4% paraformaldehyde (PFA) for 10 min. on ice, washed two times with PBS and one time with dH2O. Slides were then added to 2% (w/vol) potassium ferrocyanide (Sigma) in 1 M HCl for 20 min. at RT. After two washes in PBS, slides are counterstained for 5 min. with Nuclear Fast Red (1% (w/vol) NFRed with 5% aluminum sulfate; Sigma) and washed again with PBS then dH2O. Mounting solution (Dako) was added before the addition of coverslips and imaging using a Leica Leitz DMRB research microscope and Micro-Publisher 3.3 RTV camera. Freshly isolated LN s were embedded in OCT and snap-frozen in liquid nitrogen and stored at -80° C until sectioning. Prussian blue staining was performed with 17 μm sections that were fixed in ice-cold acetone for 1 min. Sections were then mixed for 30 min. at 37° C in 2% (w/v) potassium ferrocyanide (Sigma) in 0.5 M HCl. After thorough washing in water, sections were counter stained in 0.1% (w/v) Nuclear Fast Red (Sigma) with 5% aluminum sulfate. Slides were then mounted with Dako fluorescent mounting media for immediate imaging using a Leica Leitz DMRB research microscope and Micro-Publisher 3.3 RTV camera. Images were analyzed with Image-Pro Plus 7.0 from Media Cybernetics software and Photoshop.

**Statistics**

A t-test was performed to determine if there was a significant difference in percentage growth of LNs, in vivo proliferation or the dividing cells have undertaken. It was calculated by dividing the total number of events by the calculated number of total parent cells.

**Results**

DCs can be efficiently labelled with SPIOs while maintaining their viability

In order to identify the optimal conditions for SPIO labelling of DCs using the clinically approved SPIO Endorem, various iron concentrations (50, 100, and 150 μg/mL) and incubation times (4 to 18 h) were investigated. No adverse effects were observed for all labelling conditions using both trypan blue and MTT assays. Due to previous literature reports of decreased viability, phagocytic function, and mobility at higher concentrations of SPIO and longer incubation times, SPIO labelling was performed at 100 μg/mL of Endorem for 4 h at 37°C [3,6]. The average concentration of iron per cell under this labelling condition was determined by the ferrozine-based spectrophotometric iron quantification assay and showed an average of 7 pg iron per cell. The amount of iron in the cells was evaluated by using the Prussian blue stain (Figure S1). As reported before, the SPIO labelling was heterogeneous and some cells were heavily labelled while some not at all [4]. However, prussian blue staining revealed that >90% of DCs were stained blue for iron after SPIO labelling as seen by the presence of any blue stain in the DC under high magnification. Standard transmission electron microscopy (STEM) was performed to further analyse intracellular iron. Unlabelled cells did not show dark endosomal-like compartments as seen with SPIO labelled DCs (Figure 1A–C). To confirm that these dark regions within the DCs were SPIOs, energy filtered transmission electron microscopy (EFTEM) was used to map the iron and oxygen distribution within SPIO labelled DCs. Both the EFTEM analysis for oxygen and iron showed similar cellular localisation results (Figure 1D and E).

To ensure that the SPIO labelling condition changed the contrast in an MR image, SPIO DCs were set in gelatin at decreasing concentrations, imaged in a 1.5 T MRI scanner and compared to control unlabelled DCs (Figure 1F). At a TE of 22.5 ms, SPIO labelled DCs at 2.5 x10^6 cells/mL were more hypointense compared to control unlabelled DCs. Utilising R2, R2' and R2 maps, intracellular iron uptake was confirmed in a phantom experiment (Figure S2A-C) [13]. The difference between R2 and R2’ mapping was used to determine R2*, which increases in the presence of intracellular iron. The R2 and R2’ maps were used to show an increase in R2’ values from SPIO DCs as compared to free SPIO in gelatin (Figure S2D).

**SPIO labelling did not affect the phenotype and in vitro function of DCs**

The phenotype of SPIO DCs after maturation with LPS was analyzed by flow cytometry and compared to unlabelled DCs. Analysis of the common DC surface markers such as MHC-class I, MHC-class II, CD11c, CD40, CD80, CD86, CD54 and CCR7 showed that the phenotype of DCs with and without SPIO labelling was very similar (Figure S3). The functionality of SPIO DCs was analyzed firstly by assessing their T cell stimulatory capacity in vitro. SPIO labelled and unlabelled, LPS treated DC derived from BL/6 mice were pulsed with Ova322–332 peptide and then incubated with freshly isolated CD4+ T cells from OT-II mice (transgenic mice with a TCR specific for Ova, peptide 322–332, and restricted by H-2A b) at a ratio of 1:10. The functionality of SPIO DCs was tested in MLR (Figure 2A). SPIO labelling of DCs did not influence OT-II CD4+ T cell proliferation. To test for the ability of SPIO DCs to retain efficient antigen processing and presentation functions, DCs were pulsed with the whole Ova protein for 4 h, prior to LPS maturation and SPIO labelling. DCs were then incubated with freshly isolated OT-II CD4+ T cells (Figure 2B). As presented in Figure 2B there was no change in the ability of SPIO DCs to stimulate antigen-specific CD4+ T cells. The functional consequence of SPIO labelling of DCs was also tested in MLR (Figure 2C). DCs derived from BL/6 mice were incubated with freshly isolated CD4+ T cells from CBA/Ca mice at different DC:T cell ratios. As shown in Figure 2C, SPIO labelling did not influence the stimulatory ability of DCs in a MLR.
SPIO labelling of DCs did not influence their migratory properties

To determine the effect of SPIO labelling on DC migration in vivo, mice were injected with $1 \times 10^6$ SPIO DCs in the right leg and $1 \times 10^6$ unlabelled DCs in the left leg. Mice were then imaged at 4, 24, 48, and 96 h post-DC injection (Figure 3A). This longitudinal imaging showed hypointensity as early as 24 h post-injection in the right popliteal LN as well as the original injection site. The hypointensity increased from 24 to 48 h post-DC injection. At 96 h, the hypointensity of iron in the right popliteal LN decreased. Furthermore, there was no significant difference in percentage LN increase between the right (SPIO labelled) and left popliteal LNs (unlabelled) ($P = 0.1888$). This indicated that in vivo there is no difference in migration and immune activation between unlabelled and SPIO labelled DCs.

Gradient echo MRI with longer echo times TE were used for visualization of iron in the popliteal LNs due to SPIO DC migration. MR Images of mice at 48 h post-DC injection with echo times of 4.6, 9.2, and 13.8 ms are shown in Figure S4A, B and C, respectively. The left popliteal LN, where unlabelled DCs were injected, showed no noticeable decrease in signal intensity with increase in TE. In fact, the LNs retain the “white” contrast while most of the signal was lost in the surrounding tissue. In the right LN, however, SPIO DCs caused a clear hypointensity present at 4.6 ms and increased at longer TEs.

As a second method for the detection of SPIO-labelled DCs the SUMO-technique was used for visualization with positive contrast (Figure 4). In both LNs there was positive contrast around the LNs.
to changes in tissue density and susceptibility. In popliteal LNs that have SPIO DCs injected, there was a region of positive contrast in the centre of the LN (Figure 4B) whereas unlabelled DCs caused minimal positive contrast in the centre of the LN (Figure 4C). Furthermore, quantitative information over the longitudinal study was obtained by measurement of the susceptibility gradient, \( G_s \), from the SUMO parameter maps (Figure 4D) and \( G_s \) was significantly higher for SPIO labelled DCs than unlabelled DC (\( P<0.038 \)).

From the periphery, DCs enter afferent lymphatic vessels to migrate to and enter the LNs. Upon entrance to the LN they reside in the subcapsular sinus before migrating to the cortex of the LN where they present antigen to lymphocytes. To confirm the presence of iron in popliteal LNs after SPIO labelled DC were injected, as shown in the MRI images; prussian blue staining with a nuclear fast red counter-stain was performed on tissue cryosections (Figure S5). Popliteal LN samples taken 48 h after SPIO labelled DC injection reveals iron in both the subcapsular sinus and in the centre of the popliteal LN.

**SPIO DCs induced in vivo T cell proliferation and protected against tumour challenge**

If SPIO DCs are to be used to monitor adoptively transferred DCs in the clinic, then it is important to establish whether SPIO DCs can migrate and stimulate T cell proliferation in vivo in an equivalent manner to unlabelled DCs. To test this, mice initially received naïve CFSE\(^+\) OT-II CD4\(^+\) T cells followed 24 hours later by DC injection. Popliteal and mesenteric LNs were collected for individual analysis by flow cytometry 4 days after DC injections (Figure 5). When mature, Ova\(_{257–264}\) pulsed DCs were injected subcutaneously, proliferation was observed in the popliteal LN as seen by CFSE dilution of CFSE\(^+\) OT-II CD4\(^+\) T cells (Figure 5A). As expected, in the mesenteric LN little proliferation was observed (Figure 5B). Flow cytometry histograms of cells from three mice showed T cell proliferation in popliteal LNs when DCs were Ova\(_{257–264}\) pulsed and not mesenteric LNs (Figure 5C). As a control, no proliferation was seen when injected DCs were not pulsed with Ova\(_{322–332}\) peptide. The percentage of original CFSE\(^+\) OT-II CD4\(^+\) T cells dividing in the popliteal LN was 42.5 ± 1.3\% and 40.1 ± 1.1\% in unlabelled and labelled DC, respectively, while in the control mesenteric LN it was 9.9 ± 0.3\% (data not shown). Similarly, the proliferation index (PI) of unlabelled and SPIO labelled DCs was 2.5 ± 0.04 and 2.3 ± 0.1, respectively, whereas the control mesenteric LN gave rise to a PI of 1.2 ± 2.1\times 10^{-6}\) (Figure 5D). Therefore, there was no significant difference between SPIO labelled and unlabelled DCs (\( P<0.061 \)) in the ability to initiate T cell proliferation in the popliteal LN.

To further investigate whether SPIO labelling can affect DC function in vivo a tumour model was used. Mice were vaccinated in both heels with 1\(\times 10^6\) mature Ova\(_{257–264}\) pulsed DCs that were either SPIO labelled or unlabelled. Four days later, mice were challenged i.v. with 1.5\(\times 10^6\) B16-Ova melanoma cells. Fourteen days later, mice were sacrificed and surface lung nodules were counted (Figure 6). Mature, Ova\(_{257–264}\) pulsed, SPIO labelled and unlabelled DCs reduced the average number of tumour nodules to 1.0±1.4 and 3.2±1.9, respectively, per lung as compared to control mice with no therapy that had >250 nodules per lung (Figure 6). A statistical significant difference in distribution of tumour nodule counts was found between all four groups (\( p<0.0001 \)). Importantly, no significant difference in the efficacy of tumour vaccination was detected between SPIO DCs + Ova\(_{257–264}\) (labelled) and DCs + Ova\(_{257–264}\) (unlabelled) (\( P>0.05 \)) and both were significantly different from control or SPIO DC (\( P<0.001 \)).

**Discussion**

In this manuscript we have shown that SPIO labelling of DCs, using the conditions selected in this study, (i) does not affect the viability and phenotype of DCs; (ii) does not affect their stimulatory capacity in vitro and in vivo; (iii) allows the visualization of SPIO DC migration to the popliteal LNs over time and (iv) allows the visualization of DC contribution to anti-tumour responses. Altogether these results support the use of MRI to follow DCs injected in vivo for either the induction of an anti-tumour response or for their capacity, when “tolerogenic,” to prevent allograft rejection or autoimmune diseases.

Recent reports in the stem cell field, comparing SPIO labelling and reporter gene imaging of transplanted stem cells in the myocardium, have shown that while hypointensity due to SPIO is retained in the heart for up to 40 days, reporter gene imaging of the same cells is gone after 3 days [15,16,17]. SPIO was found to be localized in macrophages while no stem cells were present in histological analysis. These results show that while MRI can be used to track cells, the presence of SPIO can lead to false results due to their retention in tissue by other cell types. However, here we use mature DCs that show active migration from the periphery to the popliteal LN. Moreover, the life span of an activated or mature dendritic cell in vivo, while somewhat varying according to type and origin, is only a few days and therefore the use of SPIO to MR image DCs over a few days seems appropriate. Furthermore, we predicted that if the signal reduction resulted from free SPIO engulfed by host macrophages or DCs, this signal would have only been observed at the subcapsular region of the LN and not in both the subcapsular sinus and the centre of the popliteal LN as we observed [4,18].

SPIO labelling of cells together with MRI can offer many advantages. As shown here MRI can reveal that the migration of mature, activated DCs to a LN caused LN growth or swelling. Furthermore, the strongest SPIO effect of hypointensity occurred 24–48 h after DC injection and was similar to previously reported results [4]. A recent publication has suggested that vaccination using SPIO labelled dying tumour cells allowed the imaging of endogenous DCs trafficking following the engulfment of the SPIO labelled tumour cells [18]. The signal in the popliteal LN was first detected day three post-tumour injection and reached a maximum at day 8 [18]. Therefore, in this study, the hypointensity in the popliteal LN was due to the migration of the injected cells and not host DCs that engulf the SPIO labelled DCs and subsequently migrate. In a more recent study, MPIO labelled DCs caused an
increased hypointensity for up to 7 days post-injection [9]. However, it was reported that at day 7, there were 50% less fluorescent DCs present in the LN than at day 2. This could be due in part to host DCs and macrophages engulfing and trafficking MPIO to the LN or to the slow clearance of MPIO from the LN after DC death in the LN [9].

The SUMO positive contrast technique together with imaging with varying TEs are sensitive techniques that can be used not only for DC migration but other cell tracking studies using SPIOs. SUMO allows the viewer to see dark pixels in an image due to susceptibility more easily and might also be used to provide quantitative information [19]. Using SPIO labelled DCs it can show the presence intra-nodal as opposed to the supcapsular sinus.

The creation of $T_2^*$ maps from acquisition at different TEs has the potential to be used for quantification of iron $in$ vivo. Using the signal intensity at every voxel at different TEs allows a signal decay curve to be used to produce a map that relates to the iron concentration. This was shown in phantom experiments and was also used to confirm compartmentalization of SPIO by DCs (Figure S2). In the $in$ vivo DC migration study, however, the creation of a map was difficult because the signal intensity in the popliteal LN at a TE of 4.6 ms had already decayed. The effect of the iron resulted in $T_2^*$ relaxation times too short for accurate measurement in the popliteal LN. This is due to too many SPIO labelled DCs migrating to the LN resulting in strong hypointensity at short TEs. It is possible to acquire MR images at shorter TEs to help produce a $T_2^*$ map, but it is not possible with the high-resolution parameters used in this MR acquisition. However, it would also be possible to titrate the number of SPIO labelled DCs injected so that quantification of DC migration is possible.

**Figure 3. In vivo MR imaging of SPIO DCs.** $1 \times 10^6$ SPIO labelled or unlabelled DCs were injected s.c. in the right or left heel, respectively. **A** The region encompassing the popliteal LNs was imaged serially over 96 h to observe the increase in size of both popliteal LN and detect hypointensity in the right popliteal LN. **B** The growth of the popliteal LNs over time after injection of SPIO labelled or unlabelled DCs was analysed as the percentage increase in volume compared to the volume of the LN at 4 h post-injection. The percentage increase in LN volume was not significantly different between SPIO labelled and unlabelled DCs ($P = 0.1888$). Shown is a representative example of one experiment expressed as the average of triplicate ± SE. doi:10.1371/journal.pone.0019662.g003

**Figure 4. SPIO positive contrast in LN.** $1 \times 10^6$ SPIO labelled or unlabelled DCs were injected s.c. in the right or left heel, respectively. **A** MR image at 48 h post-DC injection. Positive contrast images by SUMO at 48 h post-DC injection provide direct comparison of SPIO labelled and unlabelled DCs are compared in **B** and **C**, respectively. **D** Measurement of the susceptibility gradient, $G_s$ in LNs from SUMO parameter maps over the longitudinal study indicates that $G_s$ was significantly higher for SPIO labelled DCs than unlabelled DC ($P = 0.038$). doi:10.1371/journal.pone.0019662.g004
In this study we focused on SPIO labelling of DCs and we demonstrated that SPIO had no drastic effect on in vivo DC function, including: (i) the growth of popliteal LNs after s.c. DC injection, (ii) the PI and % original dividing cells in the in vivo proliferation assay, and (iii) the ability of DCs to protect mice from tumour challenge in the B16-Ova melanoma lung metastases model. Previous reports using an ex vivo proliferation assay stated that SPIO labelling of DCs affects the proliferation of T cells [4,6]. Dekaban et al. showed that the endocytic function was decreased in SPIO labelled DCs when KLH was used as a whole antigen [6]. In the in vivo proliferation assay described here, the Ova peptide is used instead of whole Ova protein. This negates the effect of the DCs having to engulf intact antigen. Therefore, the assay does not monitor for the processing and presenting capabilities of DCs. In order to avoid the problem of antigen pulsing, other studies have shown that transfection of DCs to express tumour specific antigens improves DC based tumour vaccination [20].

The B16 murine melanoma tumour model was used to compare the effect of SPIO labelling on DC vaccination capabilities. It should be noted, however, that both the B16 cells and ex vivo expanded DCs were cultured in media that contains FBS. Due to this, it is possible that ‘bystander’ immune responses could be elicited by the DCs against components of the FBS that are expressed on B16 tumour cells. This can lead to an anti-tumour effect and could be the reason for the decrease in lung nodules when SPIO labelled DCs not pulsed with Ova257-264 were used as a control for DC vaccination. This effect of FBS culture conditions leading to anti-tumour immune responses in the B16 model has been reported in previous studies [21,22]. Eggert et al. reported that bone marrow derived DCs, either unloaded or tumour-associated antigen loaded, cultured in FBS could protect against a challenge with i.v. or s.c. injected B78-D14, a variant of B16 [21]. However, DCs without FBS in the culture media only led to protection against B78-D14 when they were loaded with the TRP-2 peptide of amino acids 180–188 [21]. Toldbod et al. reported similar results of the antitumour effect of FBS cultured DCs upon B16 challenge that had also been cultured in FBS [22]. In the B16 model described here, the antigen unpulsed DCs resulted in the presence of a range of tumour nodules when challenged with B16 tumour injection and SPIO labelling procedure did not effect the ability of Ova257-264 pulsed DCs to completely eliminate Ova 257-264 expressing tumour cells. However, the possibility remains that uptake of dead SPIO DCs or DC exosomes by lymph node resident DCs, which then act as antigen presenting cells cannot be ruled out.

In conclusion we have demonstrated for the first time a direct comparison of SPIO DCs to their unlabelled counterparts in vivo. The conditions described here for SPIO labelling of DCs

Figure 5. SPIO DCs induce in vivo T cell proliferation. 3 x 10^6 CFSE labelled OT-II CD4^+ T cells were injected i.v. The next day, 1 x 10^5 ± SPIO, LPS treated, OVA_{323-333} pulsed DCs were injected s.c. heel. Four days later the mesenteric (A) and popliteal (B) LNs were harvested, stained with anti-CD4-APC and analysed by flow cytometry. (C) Regions of interest highlighted in (A) and (B) are shown as representative histograms and show the proliferation peaks of CFSE labelled OT-II CD4^+ T cells in the control mesenteric LN (top panel), popliteal LN from mice which received unlabelled DCs (middle panel) and popliteal LN from mice receiving SPIO DCs (bottom panel). (D) Proliferation index ± SD after five divisions of CFSE labelled OT-II CD4^+ T cells shows specific proliferation and no significant difference proliferation in popliteal LNs from mice which received DCs pulsed with OVA_{323-333} with and without SPIO (P = 0.061). These are representative examples of three individual experiments where non-pulsed DCs are run individually and pulsed DCs are run in triplicate.
doi:10.1371/journal.pone.0019662.g005
The efficacy of tumour vaccination was detected between SPIO DCs + Shown is a representative example of three individual experiments doi:10.1371/journal.pone.0019662.g006 (TIF)

Figure S5 Prussian blue histology. 48 h post-SPIO labelled DC injection, popliteal LNs were isolated and snap frozen in OCT. A) Prussian blue staining reveals a strong iron stain in the subcapsular sinus. B) A zoom image in the center of the image reveals the presence of iron in the center of the LN. C) Prussian blue stain of SPIO unlabelled DCs in popliteal LN. D) Single cell in popliteal LN after SPIO labelled DC injection. (TIF)

Author Contributions
Conceived and designed the experiments: RT GM PS GL RS RR RL GV. Performed the experiments: RT SP GV AW. Analyzed the data: RT GM SP GL. Contributed reagents/materials/analysis tools: RT TS SD YT. Wrote the paper: GM RT SP GL.

References


