Functional assessment of human dendritic cells labeled for in vivo 19F magnetic resonance imaging cell tracking

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Abstract

Background aims. Dendritic cells (DC) are increasingly being used as cellular vaccines to treat cancer and infectious diseases. While there have been some promising results in early clinical trials using DC-based vaccines, the inability to visualize non-invasively the location, migration and fate of cells once adoptively transferred into patients is often cited as a limiting factor in the advancement of these therapies. A novel perfluoropolyether (PFPE) tracer agent was used to label human DC ex vivo for the purpose of tracking the cells in vivo by 19F magnetic resonance imaging (MRI). We provide an assessment of this technology and examine its impact on the health and function of the DC. Methods. Monocyte-derived DC were labeled with PFPE and then assessed. Cell viability was determined by examining cell membrane integrity and mitochondrial lipid content. Immunostaining and flow cytometry were used to measure surface antigen expression of DC maturation markers. Functional tests included bioassays for interleukin (IL)-12p70 production, T-cell stimulatory function and chemotaxis. MRI efficacy was demonstrated by inoculation of PFPE-labeled human DC into NOD-SCID mice. Results. DC were effectively labeled with PFPE without significant impact on cell viability, phenotype or function. The PFPE-labeled DC were clearly detected in vivo by 19F MRI, with mature DC being shown to migrate selectively towards draining lymph node regions within 18 h. Conclusions. This study is the first application of PFPE cell labeling and MRI cell tracking using human immunotherapeutic cells. These techniques may have significant potential for tracking therapeutic cells in future clinical trials.

Key Words: cancer, chemokine, fluorine-19 imaging, immunotherapy, nanoparticle, perfluorocarbon, perfluoropolyether

Introduction

Dendritic cells (DC) are often referred to as the 'professional' antigen-presenting cells of the immune system because of their unique ability to initiate and regulate primary T-cell responses (1). DC are strategically positioned throughout the body at anatomical sites common to pathogen entry, such as the skin, gastrointestinal tract and respiratory tract (1). In the periphery, they capture antigen and quickly become activated in response to pathogen-derived 'danger signals', as well as to endogenous factors released into the tissue environment during the assault (2,3). Acting as a bridge between innate and adaptive immunity, activated DC carry the pathogen-derived information from the periphery to draining lymph nodes, where they present processed antigen in the context of their major histocompatibility complex (MHC) class I and II molecules to activate naive CD8+ and CD4+ T cells, respectively (i.e. signal 1) (1). Along with this antigen-specific signal 1, DC also provide additional co-stimulatory and polarizing factors (signals 2 and 3, respectively) that regulate the magnitude and type of immune response needed to match effectively the particular character of the pathogen and the affected tissue (1,4).

Because of the unique and central role of DC in the immune response, DC therapeutics have covered a wide range of medical interests during the past two decades (5). The ability of DC to induce strong cell-mediated immune responses by...
promoting the effector functions of T helper (Th)-1 cells, cytotoxic T lymphocytes (CTL) and natural killer (NK) cells has positioned them at the forefront of therapeutic applications in the setting of chronic diseases such as cancer (5,6) and human immunodeficiency virus (HIV) infection (7). In contrast, because they promote the activity of regulatory T cells (Tregs), DC also play a critical role in the development of immune tolerance and immunosuppression (8) and thus have been a major focus of research in areas of autoimmunity as well as organ transplantation (9).

In recent years, there have been many DC-based clinical trials initiated, most notably in the setting of cancer, where activated DC are loaded with tumor antigens and utilized as anti-cancer vaccines (5,6). While protective and therapeutic responses to tumors have been observed widely in animal models (10–12), results from human clinical trials have been mixed (6,13). As Engell-Noerregaard et al. (6) reported in a comprehensive review of 38 published clinical studies for the treatment of cancer (6,13), there are many variables to account for such inconsistencies in clinical outcomes, including the particular type of DC used, the methods used for DC generation and activation, the type and source of antigen used, and the route of DC administration (5,6,14). Therefore, there is a critical need to limit this variability by developing standardized methods for the preparation of effective DC vaccines, their delivery to the patient and their functional assessment once administered (15).

One critical aspect influencing the therapeutic effectiveness of antigen-carrying DC is their requirement to enter draining lymph nodes, the place where they elicit robust adaptive T-cell responses (13). While the maturation status and route of administration can theoretically be used to predict the fate and effectiveness of the administered DC (16), in practice the therapeutic outcomes can be highly patient-specific. Non-efficacy is often attributed to aberrant DC trafficking or inefficient nodal delivery. Hence the ability to non-invasively monitor the actual trafficking and survival of DC in every patient is paramount (15).

A number of methods have been used to monitor transplanted cells in vivo. Optical approaches, using fluoroescently or bioluminescently tagged cells (17,18), are powerful tools in certain pre-clinical settings but generally ineffective for visualizing deep structures at high resolution because of the opacity of most mammalian tissues. Radioisotope cell tagging, using positron emission tomography (PET) or single-photon emission computed tomography (SPECT) detection, is a highly sensitive technique but has limited spatial resolution (19,20) and is complicated by the requirement of inoculation and handling of radioactive materials in the clinic. Moreover, labeled cells can only be detected for a limited time period because of a finite half-life of the radioisotope.

Alternatively, magnetic resonance imaging (MRI) techniques allow for high-resolution three-dimensional (3-D) imaging and are widely used clinically. One MRI-based technique that has been studied extensively for in vivo tracking of administered cells employs superparamagnetic iron oxide (SPIO) nanoparticles (21–23). This method has been proven useful in the clinic for monitoring human DC following their injection into lymphoid tissue (21). However, a challenge often encountered with metal ion-based contrast agents is that the large proton (1H) background signal present in soft tissues makes it difficult to identify unambiguously regions containing labeled cells throughout the body. It may be necessary to interpret subtle changes in gray-scale contrast, or measured relaxation rates, in regions believed to contain labeled cells. Consequently, cell quantification in regions of interest (ROI) is experimentally complex, perhaps requiring two MRI scans, both pre- and post-inoculation.

In this study we explored labeling human DC ex vivo with a commercially available perfluoropolyether (PFPE) tracer agent that can be used for tracking administered cells using 19F MRI or magnetic resonance spectroscopy (MRS), either in vivo or in excised tissues. Because mammalian tissues have negligible 19F content, this PFPE-based MRI technology has the benefit of yielding positive contrast images that are highly selective for labeled cells. Moreover, the PFPE reagent does not require transfection agents to label cells efficiently, even for non-phagocytic cell types, thereby minimizing cell toxicity and cell culture manipulation. While previous studies have demonstrated PFPE labeling in either immortalized cell lines or primary rodent cells (24–27), we report here the first studies using this technology platform to label and track therapeutically relevant primary cell types generated from human subjects. We demonstrate that 19F MRI is an effective way to track human DC in vivo post-administration in a xenograft model. Importantly, we also show that PFPE labeling has no significant impact on the viability, phenotype and functional properties of the cells, making this an attractive approach for exploring clinical applications. These findings provide evidence that PFPE cell tracking may be able to improve the effectiveness of monitoring cell therapy delivery in patients.
of a PFPE perfluorocarbon polymer, having a total fluorine content of 145 mg/mL. The nanoemulsion droplet size is c. 180 nm with a polydispersity of c. 0.01. Cell Sense is specifically formulated with excipients that facilitate entry of the reagent into any cell type \textit{ex vivo}, regardless of intrinsic phagocytic ability. The PFPE molecule used in Cell Sense is both lipophobic and hydrophobic, stable at low pH and is not degraded by any known cell enzyme.

To label immature and mature DC, PFPE nanoemulsion was added to the DC cultures on day 5. For mature DC, the PFPE reagent was added just prior to the addition of the cytokine maturation cocktail (29). The reagent was tested at concentrations ranging between 1 and 8 mg/mL for initial testing. Subsequently, an empirically determined optimal dose of 3 mg/mL (see Results) was used for all assays, except where indicated. The cells were exposed to the PFPE reagent under normal tissue culture conditions until day 7. Sterile water (HyClone, Logan, UT, USA) was added to control cultures at volumes equal to that of the test reagent (mock-labeled). The DC were harvested and washed three times in RPMI to remove excess labeling reagent before testing.

Cell viability

Cell viability was determined by microscopic analysis using a standard trypan blue exclusion method. Viability was also measured using the 10 N-nonyl acridine orange (NAO) assay (30) to stain and measure the loss of the mitochondrial lipid cardiolipin that occurs in early-stage apoptosis. NAO was added at a final concentration of 0.2 μM to a 1-mL cell suspension of DC containing approximately 1 × 10^6/sample at 37°C for 15 min. To induce apoptosis for positive controls 1 × 10^6 mature DC were treated with ultraviolet (UV) light 1 h prior to testing, as described previously (31). The cells were then washed and analyzed by flow cytometry using a Beckman Coulter Epics XL (Beckman Coulter Inc., Fullerton, CA, USA).

Nuclear magnetic resonance analysis of PFPE-labeled DC

To assay the 19F content of the DC after PFPE labeling, cell pellets containing a known cell number (approximately 2–3 × 10^6) were lysed with 100 μL 1% Triton X (Sigma-Aldrich). A standard 19F reference solution was added to the lysate consisting of 100 μL 0.1% (vol/vol) trifluoroacetic acid (TFA), and the mixture was placed in a 5-mm quartz nuclear magnetic resonance (NMR) tube. 19F NMR analysis on cell pellets was conducted
using a high-resolution Bruker AVANCE spectrometer (Bruker BioSpin, Billerica, MA PFPE) operating at 282 MHz. One-dimensional $^{19}$F spectra were obtained, where both PFPE and TFA appear as single narrow resonances, having a chemical shift difference of $-15.58$ p.p.m. The ratio of the integrated areas under these two peaks can be used to calculate the mean $^{19}$F/cell, often ranging from 10$^{11}$ to 10$^{12}$, as described previously (25,26).

**Stability of intracellular PFPE label**

To determine the long-term stability of the intracellular PFPE labeling, DC were monitored for fluorine content for several days. Mature DC were labeled, harvested and a sample of the cell culture assayed using $^{19}$F NMR via the same methods described above. The remaining cells were then placed back into fresh DC culture medium containing maturation factors. Subsequently, cell aliquots were harvested at various time-points over 6 days and analyzed for $^{19}$F content using NMR analysis. Three independent experiments were performed using DC cultures derived from a single donor and the results were averaged.

**Immunostaining for flow cytometry**

Two-color cell-surface immunostaining analysis was performed using a Beckman Coulter Epics XL flow cytometer. Fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled anti-human CD86 antibodies (clone 2331, Fun-1) and the corresponding (mouse IgG1) control antibodies were purchased from BD-PharMingen (San Jose, CA, USA). PE-labeled anti-human CD83 (clone HB15a) and the corresponding PE-isotype (mouse IgG2b) control monoclonal antibodies were purchased from Coulter Immunotech (Miami, FL, USA). FITC-labeled anti-human CCR7 (clone 150503) and the corresponding FITC-isotype (mouse IgG2a) monoclonal antibodies were purchased from R&D Systems and BD-PharMingen, respectively. Before staining, the cells were treated for 15 min in phosphate-buffered saline (PBS) containing 0.1% NaN$_3$, 2% human serum, 1% bovine serum albumin (BSA) and 1 $\mu$g/mL mouse IgG (Sigma-Aldrich) to block non-specific Fc receptor-binding sites. Following an incubation for 20 min at 4°C with the staining antibodies, the samples were washed with PBS containing 0.1% NaN$_3$, fixed with 2% paraformaldehyde and then stored in the dark at 4°C until the time of analysis. Data were analyzed using the WinMDI flow cytometry analysis software obtained on-line from The Scripps Institute Flow Cytometry Core Facility facs.scripps.edu/facsindex.html.

**DC production of IL-12p70**

DC were harvested, washed and plated in 96-well plates at $2 \times 10^4$ cells/well. Using a method described previously (32,33) to mimic DC interactions with CD40L-expressing Th cells, CD40L-transfected J558 cells (a gift from Dr P. Lane, University of Birmingham, Birmingham, UK) were added at $5 \times 10^4$ cells/well with or without recombinant human (rh) IFN-γ (1000 IU/mL). Supernatants were collected after 24 h and analyzed by IL-12p70 enzyme-linked immunosorbent assay (ELISA; Endogen Inc., Woburn, MA, USA).

**T-cell stimulatory capacity**

DC-mediated T-cell proliferation was assayed using a standard one-way mixed lymphocyte reaction (MLR) using human allogeneic donor T cells as responders to the stimulating DC. Briefly, purified DC were added at different ratios to responder T cells that were plated at a fixed concentration ($1 \times 10^5$ cells/well) in a 96-well flat-bottomed plate. Control wells containing only the responder T cells or DC alone were also prepared. The MLR was incubated for 96 h at 37°C and then 50 $\mu$L luminescent cell-counting reagent CellTiter-Glo® (Promega Inc., Madison, WI, USA) were added to the wells, and the assays were measured using a Victor luminescence plate reader (Perkin-Elmer Inc., Waltham, MA, USA). When reporting the data, the relative light unit (RLU) values representing the control wells were subtracted from the sample value at each DC:T-cell ratio tested.

**Chemotaxis**

**In vitro** DC migration was induced using the chemokine CCL21 (6CKine; R&D Systems) and measured using the 300-$\mu$L 96-well 8-µm pore ChemoTx® system (Neuro Probe Inc., Gaithersburg, MD, USA). First, the CCL21 was serially diluted in triplicate wells with assay medium consisting of IMDM medium containing 10% FBS and placed in the bottom receiving chambers. DC were washed, harvested and adjusted to a concentration of $1 \times 10^6$ cells/mL in IMDM medium containing 10% FBS. To obtain a maximum migration control value, 30 $\mu$L cell suspension ($3 \times 10^4$ DC) were placed in the bottom of the receiving chambers in triplicate wells. Once the pore membrane was in place, 30-$\mu$L droplets of the cell suspension ($3 \times 10^4$ DC) were placed on the top of the membrane above the serial dilutions of CCL21. The DC were then permitted to migrate for 90 min at 37°C in response to the CCL21. The amount of spontaneous migration was determined by plating cells above control wells
containing medium in the absence of CCL21. To stop the assay, the upper membrane was gently wiped free of the droplets containing cells that failed to migrate using a cotton swab. The membranes were removed and the plates were placed in a centrifuge and spun gently for 1 min to bring the cells to the bottom of the well. A volume of 50 μL media was removed from each well and replaced with 50 μL CellTiter-Glo® cell-counting reagent. The plates were incubated at room temperature for 15 min before assaying using a plate reader. The percentage of total DC migration was calculated using the formula 
\[
\frac{\text{Ns} - \text{Nc}}{\text{Nm} - \text{Nc}} \times 100\%
\]
where Ns is the mean count of a sample (migrated cells), Nc is the mean of the spontaneous control (without CCL21) and Nm is the mean count of maximum control (total cells).

**MRI**

To visualize DC in vivo, we used a xenograft mouse model. The PFPE-labeled DC (1 × 10⁶) were inoculated subcutaneously (s.c.) into the thighs of 8-week female NOD-SCID mice (n = 4). The right and left legs received mature and immature DC, respectively. Mice were imaged immediately after DC injection and then again c. 18 h after injection. Mice were anesthetized with isoflurane in air. MRI was performed at MIR Preclinical Services Inc. (Ann Arbor, MI, USA) using a Varian INOVA 7 T scanner with a 19F/1H tunable surface coil (m2m Imaging Corp., Cleveland, OH, USA). The field of view (FOV) encompassed the inguinal lymph node and the injection site. A capillary containing a 1/16 dilution of Cell Sense was placed within the FOV to provide a reference 19F signal. An anatomical 1H scan was acquired using a standard spin-echo sequence with TR/TE = 1200/15 ms, a 28 × 28 mm² FOV, a 128×128 matrix and 15 coronal slices of 0.5-mm thickness. The 19F data were acquired using the same slice parameters, except we used a 64 × 64 matrix and a fast spin-echo sequence (RARE) where eight echoes were acquired per excitation. Because of limited FOV of the surface coil, one quadriceps was imaged at a time.

To visualize and analyze the 19F images, the software program Voxel Tracker™ (Celsense Inc.) was used. This software fuses and displays 1H and 19F images; the 19F was rendered in pseudocolor using a ‘hot-iron’ scale and overlaid onto the corresponding gray-scale 1H image. Additionally, Voxel Tracker’s built-in image analysis algorithm was used to determine quantitatively the relative numbers of DC in the injection site and the draining inguinal lymph node. This published algorithm (25,26) utilizes the measured 19F/cell for the DC, as determined above, and the integrated signal from ROI in the 19F image.

**Processing of lymphoid tissue**

Post-MRI, the animals were killed and tissues containing the inguinal lymph nodes were surgically removed. Using mechanical disruption, a single-cell suspension was prepared from the lymph nodes and then passed through a 70-μm pore filter. Cells were then analyzed by flow cytometry to test for expression of human CD86.

**Statistical analysis**

A two-sided paired Student’s t-test was used to compare differences between PFPE-labeled DC to control DC across individual in vitro experiments (see above). Differences between samples were considered statistically significant when the two-sided P-value was less than 0.05.

**Results**

**Human DC can be labeled effectively with a 19F MRI tracer agent**

Using conventional NMR spectroscopy, we were able to demonstrate that human DC can be labeled effectively with PFPE (Figure 1). A representative 19F NMR spectrum (Figure 1A) displayed a single peak from the intracellular PFPE at ~91.58 p.p.m. in addition to the TFA reference at ~76.00 p.p.m. From the integrated areas of these two peaks we calculated the mean 19F/cell of the DC (Figure 1B). In Figure 1B, the 19F content/cell is shown to increase monotonically with increasing PFPE added to culture, as expected. The overall uptake levels achieved were the same order of magnitude as previous studies in murine cells (24–26). Figure 1B shows the results in mature DC. For immature DC (data not shown) the uptake behavior was qualitatively similar, except the 19F content/cell was slightly (c. 25%) higher, consistent with the known higher phagocytic activity of immature DC prior to exposure to maturation stimuli (1).

A longitudinal study was performed to test the intracellular labeling stability of DC. Mature DC are non-mitotic, thus we could monitor the 19F signal over time in cell samples taken from PFPE-labeled cultures in the absence of cell division. We found that the 19F signal from labeled DC could be detected by NMR up to 6 days following PFPE labeling (Figure 1C) with essentially no change (<1% reduction) in the cellular 19F content, indicating that the cells did not expel the PFPE. The duration of the study was limited by the relatively short lifespan of cultured DC, which was in the order of 6 days. Beyond 6 days the viability of both the PFPE-labeled and control (i.e. unlabeled) DC rapidly decreased (data not shown).
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PFPE labeling does not impact DC phenotype

The maturation status of a DC is generally defined by the expression level of various cell-surface antigens as well as the functional characteristics of the cell. Immature DC express low to moderate levels of the T-cell co-stimulatory molecule CD86 and do not express the maturation-associated molecule CD83 (34–36). Activation of DC is associated with a rapid increase in the surface expression of CD86 accompanied by the appearance of CD83 (34–36). Any changes in the expression of these two markers would probably represent alterations in DC functional capabilities. To test whether the PFPE-labeling procedure modulates the activation status of DC, we performed flow cytometric analysis to examine expression of CD86 and CD83 (Figure 3A).
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PFPE-labeled immature and mature DC were stimulated by CD40L-expressing J558 cells (see the Methods) in the absence or presence of IFN-γ. As expected, after 24 h of stimulation the CD40L-induced production of IL-12p70 by immature DC was higher than mature DC (Figure 4A). The ability of immature DC to produce IL-12p70 in response to CD40L was greatly increased (25-fold) in the presence of IFN-γ, while mature DC were not as responsive to the two-signal combination. Importantly, the amount of IL-12p70 produced by the labeled DC did not differ significantly from the amount produced by mock-labeled DC for all conditions tested (Figure 4A).

A critical aspect of DC function is the ability to induce the activation and proliferation of antigen-specific T cells. To test the T-cell stimulatory capacity, labeled DC were used as stimulators of allogeneic responder T cells in a standard MLR. As expected, mature DC induced a higher degree of T-cell proliferation than immature DC (Figure 4B). Furthermore, there were no observable differences between PFPE-labeled and mock-labeled DC (Figure 4B).

Lymph node homing capability is maintained for PFPE-labeled mature DC

In order for a DC vaccine to induce a primary T-cell response effectively, it must be capable of finding its way to the T-cell area of a draining lymph node. Expression of CCR7, the receptor for the lymph node-associated chemokines CCL19 and CCL21, is a marker of the lymph node migratory ability of mature DC (42,43). CCR7 is required

PFPE labeling does not alter DC immune function

IL-12 is a potent inducer of the activation, survival and effector functions of NK cells, CTL and CD4+ Th1 cells (37). Upon their antigen-specific interaction with CD40 ligand (CD40L)-expressing CD4+ Th cells, DC can produce IL-12p70 to promote type-1 immunity (32,38–40); this response is effective for controlling intracellular infections as well as cancer. When combined with IFN-γ, a factor produced in high amounts by activated Th1 cells, CD8+ T cells and NK cells, CD40L-induced DC production of IL-12p70 is greatly enhanced (35,41). However, upon terminal maturation, DC become ‘exhausted’ (36), producing IL-12p70 much less efficiently (35), and also become less responsive to the effects of IFN-γ (4,34).

To mimic the DC interaction with CD40L-expressing Th cells, and to analyze the impact of 19F-labeling on DC production of IL-12p70, PFPE-labeled immature and mature DC were stimulated by CD40L-expressing J558 cells (see the Methods) in the absence or presence of IFN-γ. As expected, after 24 h of stimulation the CD40L-induced production of IL-12p70 by immature DC was higher than mature DC (Figure 4A). The ability of immature DC to produce IL-12p70 in response to CD40L was greatly increased (25-fold) in the presence of IFN-γ, while mature DC were not as responsive to the two-signal combination. Importantly, the amount of IL-12p70 produced by the labeled DC did not differ significantly from the amount produced by mock-labeled DC for all conditions tested (Figure 4A).

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In vivo detection of PFPE-labeled DC by MRI

To verify that the PFPE reagent could be used as an in vivo MRI tracer for human DC, we injected PFPE-labeled DC (c. $1 \times 10^6$) into mice. We used immune-compromised NOD-SCID mice as xenograft recipients to ensure that the human DC would not be eliminated quickly by the host before images could be collected. While others have used NOD-SCID mice to study the migratory properties of human cells (44), the primary goal of our experiments was to verify that the PFPE cell labeling was efficacious for MRI, not to assess the in vivo migratory function of human DC in mice.
We administered mature and immature DC subcutaneously into the right and left thighs, respectively.

The in vivo MRI results clearly displayed DC at the injection site; Figure 6A shows typical $^{19}$F/$^1$H fusion images taken immediately and 18 h post-injection. The immature DC were not observed to leave the injection site on the contralateral side. Interestingly, however, a distinct population of the PFPE-labeled mature DC was observed located away from the injection site near the draining inguinal lymph node region at 18 h. Figure 6B shows an alternative visualization of the mature DC injection.
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DC isolated from the contralateral lymph nodes (Figure 6C). Thus PFPE-labeled mature human DC maintained their in vivo migratory function in this xenograft rodent model.

Discussion

In recent years there has been significant progress in the field of cancer immunotherapy. Clinical research has evolved from implementing ‘passive’ immunotherapeutic approaches, such as the infusion of large numbers of effector NK cells or antigen-specific CTL, to more ‘active’ immunotherapies where attempts are made to induce specific immune responses and effector cell functions within the patient. Active immunotherapies often involve administering relatively small numbers of antigen-loaded DC that act as anti-tumor vaccine carriers (13). Unfortunately, there is generally no accepted biomarker that can be used to verify whether the
administered cells ever find their intended target in vivo unless an obvious clinical response is observed. Sensitive techniques such as ELISPOT and tetramer assays using peripheral blood can provide researchers with surrogate indicators of immune functions. However, blood-based assays fail to monitor the fate of the actual therapeutic cells post-administration, and rarely provide data that correlate with a therapeutic effect (13,45). There is often a large degree of variability in therapeutic outcomes among patients, and patient-specific cell trafficking patterns are often cited as being important for therapeutic efficacy. Thus there is a large gap between the cell trafficking biomarkers that exist today and what is actually needed in the field to accelerate trials. The non-invasive imaging platform described herein potentially offers a useful method for monitoring therapeutic cells at multiple time-points within their functional environment.

Because DC have such potent immunostimulatory properties, and can be generated from peripheral blood precursors using relatively simple methods, their use has become common in clinical trials (5,6). An important characteristic of DC is their innate ability to sense subtle changes in their environment, becoming easily activated with minimal exposure to danger signals (46). This study shows that, even after long-term exposure to the PFPE emulsion, DC maintain their viability and show no signs of activation or suppression of putative therapeutic properties. The PFPE-labeled DC respond appropriately to maturation-inducing stimuli, express normal levels of co-stimulatory molecules, maintain their T-cell stimulatory and IL-12p70-producing capacity, and are able to migrate in response to lymph node-associated chemokines. Overall, our in vitro studies suggest that the PFPE reagent is not viewed by DC as ‘dangerous’ to the body.

In fact, several classes of perfluorocarbon molecules have been approved by the Food and Drug Administration (FDA) for study in clinical trials as artificial blood substitutes because of their oxygen-carrying properties (47). However, none of these products is formulated for labeling cells in culture. More importantly, the NMR properties of all commercially available perfluorocarbon emulsions are far from ideal from an MRI perspective: all have multiple $^{19}$F NMR peaks in their spectra, resulting in chemical shift artifacts and/or greatly degraded sensitivity. The Cell Sense formulation is unique because it is designed to label cells in culture without transfection agents and has molecular properties that are advantageous for MRI, i.e. a single narrow line in the $^{19}$F NMR spectrum and a relatively short $T_2/T_1$ ratio.

Besides being non-toxic and having no apparent impact on the function of the labeled cells, there are several attributes of PFPE that make it attractive for in vivo cellular imaging. Perfluorocarbons are known to be highly chemically stable. They are not degraded by any known enzyme found in the body, and maintain their structure at typical lysosomal pH values (48), thereby providing long-lasting intracellular labeling. In addition, PFPE is both hydrophobic and lipophobic and does not become associated with cell membranes (48). The nanoemulsion clearance is ultimately via the reticuloendothelial system and exhalation through the lungs (49).

While there are some obvious benefits to using the PFPE approach for tracking DC, there are a number of limitations and questions that remain unanswered. Although the function of DC does not seem to be affected by PFPE labeling, it has yet to be determined whether the labeling has any impact on their antigen-processing and cross-presentation capabilities. Additionally, it is unknown whether the PFPE-labeling protocol would impact on the ability to generate differentially polarized DC using the culture conditions that have been reported previously (33,50). Another concern is the possibility of unintended labeling of resident phagocytic cells that may engulf the nanoemulsion droplets as a result of exocytosis or cell death of the originally labeled cells. If a large number of these phagocytes remain in an image ROI, false-positive signals could result. We note that ongoing studies suggest that the Cell Sense reagent does not maintain its ability to label non-phagocytic cells once released from a dying cell. Furthermore, we have not observed any evidence for active exocytosis of the PFPE. While DC themselves represent a non-mitotic cell type, in mitotic cell division dilution of the $^{19}$F signal in an ROI could potentially be a limitation for long-term cell tracking studies.

While we were able to image PFPE-labeled human DC successfully using $^{19}$F MRI in a xenograph mouse model, we believe that tracking human DC in an immunologically challenged murine host is probably not biologically relevant. Instead, the model has been used merely to demonstrate that the PFPE-labeled DC can be tracked once administered. Nonetheless, differential migratory patterns were detected between immature and mature DC phenotypes.

Generally, detection of PFPE in vivo is independent of the cell or tissue type that it resides in, and the MRI technique has no tissue-depth penetration limitations. The feasibility of tracking PFPE-labeled mouse DC using $^{19}$F MRI following different routes of administration has been demonstrated previously (24). Previous studies have shown that the minimum cell detection sensitivity is of the order $10^4$–$10^5$ cells/voxel for clinical MRI systems and $10^3$–$10^4$ for high-field animal scanners (24,25,27,51). Experimental details, such as the image acquisition methods,
magnetic field strength and detector coil configuration, determine the actual sensitivity for a particular experiment. Single-voxel MRS detection of the labeled cells is expected to yield even higher sensitivity to sparse cell numbers.

19F MRI yields a positive-contrast signal from labeled cells with no background from the host, a consequence of the negligible fluorine content of tissue. This property makes image interpretation straightforward when the 19F image is fused with a conventional 1H image, providing a vivid anatomical underlay of the soft tissue (Figure 6A). Furthermore, the positive 19F signal is of great advantage for cell tracking in regions such as lungs, liver, peritoneal cavity and bone, where use of metal ion-based contrast agents can be challenging because of the low signal in these regions or pronounced intrinsic background contrast. Importantly, as the PFPE-based technology can be used to not only determine the location of the cells but also quantify the number of apparent cells in an ROI (25), this PFPE cell tracking represents a true quantitative biomarker of the type urgently needed for assessing cell therapy efficacy.

Acknowledgments/disclosure of interests

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