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Functional assessment of human dendritic cells labeled for *in vivo* ^{19}F 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 ^{19}F 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 ^{19}F 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.

Keywords

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 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 fluorescently 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 ^{19}F MRI or magnetic resonance spectroscopy (MRS), either *in vivo* or in excised tissues. Because mammalian tissues have negligible ^{19}F 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 ^{19}F 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.

Methods

Media and culture reagents

The clinically applicable serum-free AIM-V medium (Invitrogen Inc., Grand Island, NY, USA) was used to generate DC. Iscove's modified Dulbecco's medium (IMDM) (Cellgro Mediatech, Herndon, VA, USA) containing 10% heat-inactivated fetal bovine serum (FBS; Invitrogen) was used as an assay base medium. RPMI-1640 (Cellgro Mediatech) was used as the standard DC wash buffer throughout the study. The following recombinant human cytokines were used throughout the study: granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-4, tumor necrosis factor (TNF)- α , IL-1 β , IL-6 and interferon (IFN)- γ (all from R&D Systems, Minneapolis, MN, USA). Prostaglandin E₂ (PGE₂) was purchased from Sigma-Aldrich (St Louis, MO, USA).

Isolation of monocytes and lymphocytes

Whole blood products (buffy coats) obtained from healthy donors were purchased from the Central Blood Bank of Pittsburgh (Pittsburgh, PA, USA). Peripheral blood mononuclear cells (PBMC) were isolated by density-gradient separation using lymphocyte separation medium (Cellgro Mediatech). Monocytes and lymphocyte fractions were isolated from the PBMC fraction using a Percoll (Sigma-Aldrich) density-gradient separation technique, as described previously (28). T cells were isolated from the lymphocyte fractions using the negative isolation system EasySep® (StemCell Technologies Inc., Vancouver, Canada).

Generation of human DC

Human immature and mature DC were generated from monocyte precursors as described previously (29). Monocytes were cultured for 5–7 days in six-well plates (Falcon, Becton Dickinson Labware, Franklin Lakes, NJ, USA) at 2×10^6 cells/well in the presence of GM-CSF and IL-4 (both 500 IU/mL) to generate immature DC. To generate 'standard' mature DC, day-5 immature DC were exposed to the following factors for 48 h: IL-1 β (10 ng/mL), TNF- α (25 ng/mL), IL-6 (1000 U/mL) and PGE₂ (10 μ mol/L) (29).

¹⁹F-labeling of human DC

The good manufacturing practice (GMP)-grade PFPE reagent Cell Sense (Celsense Inc., Pittsburgh, PA, USA) was used to label DC. Cell Sense is an aqueous colloidal suspension (i.e. nanoemulsion) 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 *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 nano-emulsion 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 μ M 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 ^{19}F content of the DC after PFPE labeling, cell pellets containing a known cell number (approximately $2\text{--}3 \times 10^6$) were lysed with 100 μ L 1% Triton X (Sigma-Aldrich). A standard ^{19}F reference solution was added to the lysate consisting of 100 μ L 0.1% (vol/vol) trifluoroacetic acid (TFA), and the mixture was placed in a 5-mm quartz nuclear magnetic resonance (NMR) tube. ^{19}F 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% NaN₃, 2% human serum, 1% bovine serum albumin (BSA) and 1 µg/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% NaN₃, 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 2×10^4 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 5×10^4 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 (1×10^5 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 µL luminescent cell-counting reagent CellTiter-Glo® (Promega Inc., Madison, WI, USA) 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-µL 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 1×10^6 cells/mL in IMDM medium containing 10% FBS. To obtain a maximum migration control value, 30 µL cell suspension (3×10^4 DC) were placed in the bottom of the receiving chambers in triplicate wells. Once the pore membrane was put in place, 30-µL droplets of the cell suspension (3×10^4 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 μ L media was removed from each well and replaced with 50 μ L 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 $[(N_s - N_c) / (N_m - N_c)] \times 100\%$, where N_s is the mean count of a sample (migrated cells), N_c is the mean of the spontaneous control (without CCL21) and N_m 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 (1×10^6) 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 $^{19}\text{F}/^1\text{H}$ 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 ^{19}F signal. An anatomical ^1H scan was acquired using a standard spin-echo sequence with TR/TE = 1200/15 ms, a $28 \times 28 \text{ mm}^2$ FOV, a 128×128 matrix and 15 coronal slices of 0.5-mm thickness. The ^{19}F 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 ^{19}F images, the software program Voxel Tracker™ (Celsense Inc.) was used. This software fuses and displays ^1H and ^{19}F images; the ^{19}F 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 ^{19}F /cell for the DC, as determined above, and the integrated signal from ROI in the ^{19}F 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- μ m 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).

DC viability is unaffected by labeling

Next we investigated whether long-term (48-h) exposure and uptake of PFPE induced any alteration in cell viability. Using the trypan blue exclusion assay we observed that, even after exposure to relatively high concentrations of PFPE, both immature and mature DC maintained comparable viability to mock-labeled control cells (Figure 2A). In addition, using the NAO assay, we were unable to observe any evidence of decreased mitochondrial lipid content typically associated with apoptotic cell death (30) (Figure 2B). Although labeling had no remarkable impact on cell viability, we did find a small decrease in overall cell yield for DC exposed to very high concentrations (4 mg/mL) of PFPE reagent (Figure 2A).

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). Importantly, these data showed that the labeling procedure did not cause a change in the normal expression levels of these markers on either immature or mature DC. Thus the PFPE reagent was not associated with any harmful or danger signals innately recognized by DC. While there were no obvious phenotypic differences between the PFPE-labeled DC versus control, there was a notable increase in the light side-scatter properties of PFPE-labeled cells, as determined by flow cytometry (Figure 3B). This was probably because of increased granularity within the cytoplasmic regions of the cells, resulting from the uptake of the PFPE emulsion droplets.

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).

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 for DC entry into

lymphatic vessels and for migration into the T-cell regions of draining lymph nodes (42,43). To determine the impact of PFPE labeling on the migratory capacity of mature DC, we examined the surface expression of CCR7 by flow cytometry. Compared with mock-labeled DC, the surface expression of CCR7 for labeled DC was identical (Figure 5A). Moreover, the *in vitro* chemotactic responsiveness to the CCR7 ligand CCL21 for labeled versus mock-labeled DC remained unchanged, whereas, as expected, the CCR7-positive mature DC migrated much more effectively compared with immature DC lacking expression of CCR7 (Figure 5B).

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.* 1×10^6) into mice. We used immune-compromised NOD-SCID mice as xeno-graft 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 s.c. into the right and left thighs, respectively.

The *in vivo* MRI results clearly displayed DC at the injection site; Figure 6A shows typical $^{19}\text{F}/^1\text{H}$ 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 site and inguinal lymph node, displayed as a series of contiguous ^{19}F image slices without the ^1H underlay; cell migration to the node was readily observed. Using quantitative image analysis, we were able to determine that for animals receiving the mature DC, an average of approximately 60% (1.8×10^6) of the injected cells could be detected at the injection site, and approximately 5% (1.6×10^5) of the injected cells reached the lymph node region ($n = 3$).

To determine whether the ^{19}F signal detected in the nodal region was indeed mature human DC, we harvested the inguinal nodes from the mice ($n = 3$) to analyze the cellular contents. We prepared single-cell suspensions from this tissue and performed flow cytometry on the isolated cells to test for surface expression of the human DC marker CD86. The results from this analysis (Figure 6C) clearly showed the presence of human CD86⁺ mature DC in the draining lymph nodes. In addition, we did not observe any evidence for the presence of 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 xeno-graph 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 highfield 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.

^{19}F 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 ^{19}F image is fused with a conventional ^1H image, providing a vivid anatomical underlay of the soft tissue (Figure 6A). Furthermore, the positive ^{19}F 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.

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Acknowledgments/disclosure of interests

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Figure 1.

PFPE nanoemulsion (Cell Sense) effectively labels human monocyte-derived DC. (A) ^{19}F NMR analysis of mature DC exposed to 3 mg/mL PFPE reagent. (B) Labeling of mature DC exposed to increasing concentrations of PFPE and recorded as ^{19}F content per cell (mean of triplicate cultures $\pm$ SD). (C) Labeling persistence of mature DC cultures over a period of 6 days. These data are the average results of three labeled DC cultures, and the initial time-point (day 1) is normalized to 100%. The error bars are the standard deviation of the percentage of labeling persistence.

Figure 2.

Labeling human DC with PFPE has no impact on cell viability. (A) Cell viability of mature DC (mDC) generated from one representative donor as determined by microscopic analysis using trypan blue exclusion (mean of triplicates cultures $\pm$ SD). (B) Mitochondrial lipid assessment of mature DC using NAO staining and fluorescence-activated cell sorter (FACS) analysis. Unstained control DC are represented as the open histogram in the top panel. UV-treated DC were used as a positive control for apoptosis (bottom panel). Data are representative of four donors tested, all yielding similar results.

Figure 3.

PFPE labeling does not impact on the cell-surface phenotype of human DC. (A) Surface expression of maturation-associated co-stimulatory molecules CD86 and CD83. The shaded histogram represents PFPE-labeled DC, and mock-labeled DC are depicted by the solid line. (B) Forward- and side-scattered light from mock-labeled and PFPE-labeled mature DC as determined by flow cytometry. Data shown are from one representative experiment from a single donor. A total of four donor DC cultures were tested, all yielding similar results.

Figure 4.

PFPE-labeled DC maintain IL-12p70-producing capacity and T-cell stimulatory function.

(A) IL-12p70 production by mature DC stimulated with the Th signal CD40L alone and in combination with IFN- γ (mean of triplicate cultures $\pm$ SD). The data shown are from one ELISA assay performed on triplicate DC cultures generated from one representative donor. A total of three different donors was tested, all yielding similar results. (B) Results from a standard 5-day proliferation assay where DC were used as stimulators for responder human T cells isolated from a single allogeneic donor (mean of triplicate DC-T-cell co-cultures $\pm$ SD). iDC, immature DC. Viable T-cell counts were determined using the CellTiter-Glo $^{\text{®}}$ luminescent cell-counting assay, reported in RLU. Statistical analysis using a two-tailed paired Student's *t*-test determined that there were no statistical differences between labeled and mock-labeled samples (*P*-values >0.05).

Figure 5.

Migratory capacity of human DC is not affected by PFPE labeling. (A) Cell-surface expression of the lymph node-homing chemokine receptor CCR7 on mature DC. Data shown are representative of four similar experiments. (B) Results of the in vitro DC migratory response to the lymph node-associated chemokine CCL21 using a transwell assay. These results are from a single representative experiment out of three performed. Data are displayed as percentage cell migration (mean of triplicate wells $\pm$ SD). Statistical analysis using a paired Student's *t*-test showed no difference between labeled and mock samples (*P*-value >0.05).

Figure 6.

Detection of human DC in vivo via ^{19}F MRI in a xenograft mouse model. (A) PFPE-labeled human immature and mature DC were injected subcutaneously into the left and right quadriceps, respectively, in NOD-SCID mouse recipients. ^{19}F and ^1H images of each thigh were captured at time 0 h (top panels) and 18 h (bottom panels) post-administration. In these composite images the ^{19}F is rendered in 'hot-iron' pseudocolor and the ^1H is in gray-scale. The circled region shows the draining lymph node region. (B) Representative contiguous ^{19}F image slices through the mature DC injection site and proximal inguinal node region at 18 h post-injection. The panel displays the ^{19}F reference capillary (R), injection site (i) and the inguinal node (n), and the slice thickness is 0.5 mm. Migration of the DC from the injection site to the lymph node was clearly observed, and quantitative image analysis revealed that c. 5% of the cells reached the node. (C) Flow cytometry analysis using an anti-human CD86 marker to test for human DC presence in single-cell isolates derived from the draining lymph node tissue from the same mice. The circled region contains 0.7% of the total number of lymph node cells analyzed, where the cells from similar lymph nodes (i.e. left or right) were pooled from three mice. Data were recorded as percentage human CD86⁺ cells $\pm$ SD; nd, not detected.