19F-MRI for monitoring human NK cells in vivo

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ABSTRACT
The availability of clinical-grade cytokines and artificial antigen-presenting cells has accelerated interest in using natural killer (NK) cells as adoptive cellular therapy (ACT) for cancer. One of the technological shortcomings of translating therapies from animal models to clinical application is the inability to effectively and non-invasively track these cells after infusion in patients. We have optimized the nonradioactive isotope fluorine-19 (19F) as a means to label and track NK cells in preclinical models using magnetic resonance imaging (MRI). Human NK cells were expanded with interleukin (IL)-2 and labeled in vitro with increasing concentrations of 19F. Doses as low as 2 mg/mL 19F were detected by MRI. NK cell viability was only decreased at 8 mg/mL 19F. No effects on NK cell cytotoxicity against K562 leukemia cells were observed with 2, 4 or 8 mg/mL 19F. Higher doses of 19F, 4 mg/mL and 8 mg/mL, led to an improved 19F signal by MRI with 3 × 1011 19F atoms per NK cell. The 4 mg/mL 19F labeling had no effect on NK cell function via secretion of granzyme B or interferon gamma (IFNγ), compared to NK cells exposed to vehicle alone. 19F-labeled NK cells were detectable immediately by MRI after intratumoral injection in NSG mice and up to day 8. When 19F-labeled NK cells were injected subcutaneously, we observed a loss of signal through time at the site of injection suggesting NK cell migration to distant organs. The 19F perfluorocarbon is a safe and effective agent for monitoring the persistence and trafficking of NK cell infusions in vivo, and may have potential for developing novel imaging techniques to monitor ACT for cancer.

Introduction
The infusion of NK cells as treatment for relapsed solid tumors has been utilized by many centers based on emerging preclinical evidence of antitumor activity. Melanoma, renal cell carcinoma, recurrent breast and ovarian cancer have been treated with NK cells or NK cell lines in adults, while neuroblastoma, medulloblastoma, osteosarcoma, rhabdomyosarcoma and Ewing sarcoma are being tested in children. As NK cells are being more widely used for cancer treatment, understanding where adoptively transferred NK cells traffic after infusion is becoming more critical. It is also not known how long adoptively transferred NK cells persist in vivo, making timing of repeated NK cell infusions difficult to predict. According to the Food and Drug Administration (FDA) Cellular, Tissue and Gene Therapy Advisory Committee, there is an urgent need to track cells in vivo to determine migration patterns and longevity. A thorough summary of imaging methods that have been employed to track NK cells has been reviewed previously. Usage of superparamagnetic iron oxide (SPIO) particles have been previously used to label NK cell lines for detection in preclinical models by MRI, but have not gained wider clinical use. One limitation is that the hypointense signal produced can make it difficult to discriminate the SPIO-labeled cells from other hypointense signals, like blood, or from signal loss due to susceptibility and field inhomogeneities. 19F is a nonradioactive, 100% naturally abundant isotope of fluorine that can be formulated into perfluorocarbon nanoemulsions and simply incubated with cells in culture. Radiofrequency MRI coils can then be tuned to detect and image these fluorine atoms enabling tracking of cellular fate post-infusion.

In preclinical models, optical imaging using fluorescent and bioluminescent models have provided valuable insight into NK cell trafficking patterns after adoptive transfer, but these techniques assume murine NK cells traffic similarly to human NK cells, do not give high resolution of the live gross anatomical structures where the NK cells traffic to, and most importantly, are not available in the clinic. Radiolabeling of NK cells with clinically available isotopes, like 18F, 11C or 111In, offers high sensitivity but also lacks high resolution of anatomical structures, exposes the patient to ionizing radiation, and is limited by radioactive decay of the isotope (typically hours to days) preventing detection of long-term NK cell persistence. Plus, the FDA’s Center for Devices and Radiological Health has launched an initiative to reduce unnecessary radiation exposure from medical imaging.

In the past decade, very promising MRI techniques have been developed to monitor and quantify immune cells in vivo using the non-radioactive nucleus 19F as an MRI contrast agent. Because there are trace amounts of fluorine in the body (mainly in the bone matrix and teeth) that exhibit a very
short spin–spin relaxation time, there is minimal background “noise” and infusions of 19F-labeled cells could be easily identified at a high contrast to noise ratio. With a high gyromagnetic ratio similar to that of 1H, 19F has favorable physical properties that improve inherent receptivity and sensitivity, requiring only millimolar quantities per voxel for detection. Furthermore, 19F is non-radioactive, non-invasive; deep injection since used by MRI and has no known enzyme for its degradation in vivo (cleared by exhalation or by endoreticulum). Moreover, perfluorocarbon liquids are chemically and biologically inert, and no toxicity has ever been reported using 19F-labeled cells, even at large doses. To date, investigators have successfully used this approach to track murine macrophages, murine and human dendritic cells, murine T cells, hematopoietic, both murine and human neural stem cells as well as murine and human mesenchymal stem cells in preclinical models. Interestingly, 19F has been FDA approved in a phase I clinical trial to monitor a DC vaccine in colorectal patients. Initial results from three patients have recently been published showing the ability to detect human DCs by 19F-MRI without apparent toxicity. However, NK cells have been challenging to label using this approach and data is lacking on NK cell trafficking even though they are being infused in clinical trials.

In this study, we will show the impact of labeling human NK cells with a commercially available 19F-perfluoropolyether (PFPE) nanoemulsion on viability, cytotoxicity and cell surface marker expression on in vitro human NK cell cultures. We will also show that MRI can detect 19F-labeled human NK cells after subcutaneous and intratumoral injection. Our preliminary results indicate that 19F MRI is a promising approach for tracking NK cells in vivo noninvasively without toxicity or ionizing radiation, and has sufficient resolution to detect NK cells within a solid tumor.

Results

Labeling of human NK cells with 19F

We first determined the impact of labeling IL-2 expanded NK cells ex vivo with 19F by analyzing NK cell viability with increasing concentrations of the tracer. We found no impact on cell viability after 24 h incubation with 2 or 4 mg/mL 19F, compared to unlabeled NK cells (Fig. 1A). Viability of NK cells was only decreased at 8 mg/mL 19F (Fig. 1A). Using NMR, we detected up to $3.78 \times 10^{11}$ atoms of 19F per NK cell on average, which plateaued between labeling with 4 to 8 mg/mL 19F (Fig. 1B–C). Based on the similar efficiency of internalization of 19F using 4 mg/mL or 8 mg/mL, and since NK cell viability is decreased with 8 mg/mL 19F, we decided to use 4 mg/mL 19F for all subsequent experiments.

NK cell receptor expression upon 19F labeling

We then evaluated the effects of 19F labeling on CD3−CD56+ NK cell surface receptors expression, particularly those that are involved in NK cell antitumor activity. There were no differences in natural cytotoxicity receptor (NCR) expression in NKP30, NKP46 and NKP44 expression between unlabeled and 19F-labeled NK cells for 24 h (Fig. 2A–B). Additionally, no changes were noted in the activating receptors DNAM-1 or NKG2D and in CX3CR1 and CXCR4 chemokine receptor expression both in percentages (Fig. 2C–E) and in mean fluorescent intensity (MFI) (Fig. 2F–G).

In vitro cytotoxicity of 19F-labeled NK cells

While our flow cytometry data demonstrates that 19F does not negatively impact the expression of NK cell cytotoxicity receptors, we next tested the impact of 19F labeling on NK cell tumor cytotoxicity against K562 cells. There were no differences in NK cell cytotoxicity between unlabeled and NK cells labeled with 2 mg/mL, 4 mg/mL or 8 mg/mL 19F PFPE (Fig. 3A). We next evaluated NK cell function after in vitro stimulation by measuring intracellular production of IFNγ and granzyme B, which are both essential mediators in tumor lysis. By comparing unlabeled versus 19F-labeled NK cells, we found no difference in granzyme B production in percentage and MFI (Fig. 3B–D), whereas a mild increase in the percent and MFI of NK cells secreting IFNγ was observed in 19F-labeled NK cells (Fig. 3B–D). To verify this observation, we quantified IFNγ secretion by ELISA and found no differences after 24 or 48 h of 19F labeling at both 2 mg/mL and 4 mg/mL concentrations (Fig. 3E). Preservation of NK cell production of granzyme B and IFNγ after 19F uptake correlates with their in vitro ability to mediate antitumor responses.

In vivo detection of 19F-labeled NK cells by MRI

Next, we wanted to examine if 19F-labeled NK cells could be detected by in vivo MRI using a volumetric 1H/19F quadrature coil. We performed intratumoral injection of 19F-labeled NK cells into two different xenograft tumor models to determine if the NK cells could be detected within a bulky tumor mass. NK cells were detected at both 7 and 8 h after injection into immunodeficient mice bearing human neuroblastoma (Fig. 4A) as well as up to 8 d after injection into human lymphoma (Fig. 4C). On day 0, quantification of 19F signal from the cells using MRI showed detection of 95% of the NK cells injected intratumor for the neuroblastoma and 64% NK cells detected after 48 h (Fig. 4B). However, only 74% of the NK cells were detected on day 0 for the lymphoma-bearing mouse and the number of NK cells detected by day 8 was 68% (Fig. 4D). Overall, very little to no migration of the NK cells out of the tumor was observed and a high number of NK cells could be detected within a bulky tumor mass over the duration of the experiment (Fig. 4).
Figure 1. High uptake of $^{19}$F nanoemulsions by human NK cells do not affect their viability. Human NK cells were expanded for 12 d from PBMCs of a healthy donor and sorted on day 12 of expansion by magnetic bead isolation. Sorted NK cells (CD3$^-$CD56$^+$) were co-incubated with or without $^{19}$F (Cell Sense) for 24 h then analyzed for (A) Percent viability using Trypan blue and determined before (0 hour) and after (24 h) co-culture of NK cells with 2 mg/mL, 4 mg/mL or 8 mg/mL $^{19}$F. Data presented for 11 compiled experiments. (B) Representative NMR spectra of TFA (control) or NK cells labeled with 2 mg/mL, 4 mg/mL or 8 mg/mL PFPE show $^{19}$F signal increasing with the concentration of PFPE in cell media. (C) Concentration of $^{19}$F/NK cell determined by NMR for NK cells exposed to different concentration of PFPE for 24 h. Three replicates were set up per group. Bar graph values represent the mean ± SEM tested by a one-way ANOVA. Data representative of at least three experiments with reproducible results.

Figure 2. $^{19}$F labeling of human NK cells does not alter their surface expression of activating natural cytotoxic receptors and chemokine receptors. (A–G) Human NK cells unlabeled or labeled with 4 mg/mL $^{19}$F (Cell Sense) for 24 h analyzed by flow cytometry for: (A–B) The percent expression in the NK cytotoxic receptors (NCRs) Nkp30, Nkp46 and Nkp44 vs. IgG1 control stains. (A) Illustrates representative dot plots for each NCR and their isotype controls (IgG1) for unlabeled NK cells or NK cells labeled with $^{19}$F. (B) Shows percentage of Nkp30, Nkp46 and Nkp44 on the NK cells (CD3 negative CD56$^+$) in five healthy donors. (C–E) The percent expression or (F–G) mean fluorescent intensity (MFI) in the activating receptors DNAM-1 (DNA Accessory Molecule-1) and NKG2D and in chemokine receptors CX3CR1 and CXCR4 compared to isotype controls in $^{19}$F-labeled or unlabeled NK cells. (D) Percent expression or (G) MFI in the chemokine receptors CX3CR1, CXCR4 or isotype controls after gating on the of CD3neg CD56$^+$ NK cells. MFI numbers are indicated within the histograms with color-coded MFIs indicated in the legend and corresponding to the histograms. (E) Shows the percent and (F) MFI in CX3CR1, CXCR4, DNAM-1 and NKG2D on NK cells from five healthy donors labeled or not with $^{19}$F. All gates and histograms are pre-gated on CD3$^-$CD56$^+$ NK cells. Bar graph values represent the mean ± SEM tested by two-way ANOVA. Data representative of at least four independent experiments with reproducible results.
Migration of $^{19}$F-labeled NK cells in vivo

Finally, we sought to determine the ability of $^{19}$F-labeled NK cells to migrate to a distant tumor. To address this, $^{19}$F-labeled NK cells were injected subcutaneously into the contralateral flank of mice bearing human melanoma (M21). In order to aid in the migration of NK cells to the tumor site, the hu14.18-IL-2 immunocytokine was injected intratumorally (50 μg/50 μL) on days 0–2 and 7–9 post NK cell infusion in addition to intraperitoneal injection of recombinant human IL-2 (1 $\times$ 10^6 IU/0.2 mL) on days 0 and 7 post NK cell infusion. M21 tumors express the disialoganglioside GD2, while the hu14.18-IL18 immunocytokine consists of a GD2-specific monoclonal antibody (mAB) fused to human IL-2 able to recognize and specifically bind to GD2^+ tumors. The immunocytokine has shown antitumor effects in vivo in mouse models in a phase I/II clinical trial in pediatric patients with relapsed/refractory neuroblastoma and in patients with metastatic melanoma. Therefore, we expected that GD2^+ tumor and the treatment with hu14.18-IL2 would recruit NK cells to mediate ADCC at the tumor. At day 0, 10 $\times$ 10^6 $^{19}$F-NK cells were implanted subcutaneously (flank opposite to the tumor) with on average 9.1 $\times$ 10^6 NK cells detected by MRI on day 0 (Fig. 5A–B). Over time we observed a progressive decrease in $^{19}$F signal from the NK cells with about 65% of the NK cells or $^{19}$F signal remaining at the site of injection by day 15 (Figs. 5A–B). The decrease in $^{19}$F signal is indicative of the migration of the NK cells out of the inoculation site to distant organs. However, the $^{19}$F signal from the NK cells was not detected at the tumor site or at other organs by MRI (Fig. 5A).

Discussion

Infusions of NK cells for cancer immunotherapy represent an evolving approach both for solid and hematologic cancers and are already occurring at centers around the world. Above all, few toxicities have been reported following adoptive transfer of NK cells. However, very little is known about the migration, persistence and biodistribution of NK cells post infusion. In this paper, we show for the first time that ex vivo expanded human NK cells can be efficiently labeled with $^{19}$F, enabling their in vivo detection and quantitation by MRI in immunodeficient mice and within the dense architecture of a solid tumor. Importantly, $^{19}$F labeling of human NK cells does not alter their viability, their expression of NCRs, cytokines and granzymes necessary for their cytotoxic functions. Although NK cells have shown antitumor effects in preclinical models, their full therapeutic potential in the clinic has yet to be optimized. Typically, efficacy of cellular therapy is evaluated on the basis of survival rate and tumor regression assessed for weeks and months post treatment. However, lack of response cannot be positively attributed to the inefficacy of NK
Figure 4. MRI in vivo detection of $^{19}$F-labeled human NK cells in NSG mice bearing xenograft human tumors. (A–B) $10 \times 10^6$ $^{19}$F-labeled (4 mg/mL PFPE for 24 h) human NK cells were injected intratumor into one NSG mouse bearing a human neuroblastoma (CHLA-20) on the right flank (T: Tumor). $^{19}$F intensity from NK cells or from the reference (Ref: Reference vial of $^{19}$F) vial is displayed on a “hot-iron” scale. Mouse was imaged for $^1$H and $^{19}$F by MRI using a volumetric coil and anesthetized using ketamine and xylazine. (A) Composite $^{19}$F/$^1$H images at 0 or 2 d post NK cell injection are shown with 32 min and 42 min of scan time for each day respectively. (B) The number (black bars corresponds to left y-axis) and percentage (red line graph corresponds to the right y-axis) of NK detected in the tumor is denoted and was determined based on the efficiency of $^{19}$F uptake by NK cells from NMR analysis. (C–D) $7 \times 10^6$ $^{19}$F-labeled (4 mg/mL PFPE for 24 h) NK cells were injected intratumor into one NSG mouse bearing a human mantle cell lymphoma (Z138) on the right flank. (C) Imaging for $^1$H and $^{19}$F was established as described in (A) with scan time of 42 min for each time point. (D) Number (black bars corresponds to left y-axis) and percentage (red line graph corresponds to the right y-axis) of NK cells detected at the tumor site is denoted for each imaging time point. Values represent the mean ± SEM of one single experiment. The uncertainty in the $^{19}$F reference mean signal ($\sigma_{\text{Ref}}$) was estimated as the standard deviation of the ROI drawn on the reference (see methods for quantification of $^{19}$F signal).

Figure 5. $^{19}$F-labeled NK cell migration in NSG mice bearing human GD2⁺ melanoma and hu14.18-IL-2 treatment. $10 \times 10^6$ $^{19}$F-labeled (4 mg/mL PFPE for 24 h) human NK cells were injected on the left flank subcutaneously into NSG mice ($n = 2$) bearing human melanoma (M21) tumor on the right flank (T: Tumor). On days 0–2 and 7–9, 50 μg/50 μL hu14.18-IL-2 immunocytokine was injected i.t. On days 0 and 7, $1 \times 10^6$ IU/0.2 mL rh-IL-2 was injected i.p. (A) Mice were imaged for $^1$H and $^{19}$F by MRI for 42 min at different time points. Here, a T2-weighted $^1$H image was acquired for enhanced tumor visualization. Composite $^{19}$F/$^1$H images are depicted for 1 mouse. (B) Illustrates the number of NK cells remaining at the site of injection at each time point for both mice. Day 0 refers to the day of implantation of $^{19}$F-labeled human NK cells. Values represent the mean ± SEM of one single experiment, $n = 2$. 
cells without direct measurement of the location of NK cells in respect to the tumor site and also their persistence post adoptive transfer. The lack of response to NK cell immunotherapy could simply be due to their inability to migrate to the tumor, to a low concentration of transferred NK cells or to a need of multiple adoptive transfers, all of which are dependent on the tumor type (solid vs. blood-borne), the stage of cancer and resistance of the tumor. Presently, we rely on non-specific and insensitive blood draws and bone marrow biopsies to locate infused NK cells by flow cytometry and immunohistochemistry, even though the NK cells may be in secondary lymphoid tissues or in the tumor. Moreover, biopsy of tumors is a difficult and invasive procedure that may lead to the potential of sampling error and risk to the patient. Thus, the field of cellular immunotherapy is in need of a means by which to non-invasively track infused NK cells in both normal organs and tumors.

Usage of $^{19}$F is an appealing labeling approach for in vivo imaging of NK cells (or other cell therapies) because (1) it is the only naturally occurring isotope of fluorine and does not decay, (2) does not require phagocytosis to enter the cell, (3) is a component of the chemical structure of over a dozen FDA-approved drugs and thus has a long safety record in humans, and (4) perfluorocarbons have already been successfully used in clinical trials for decades in artificial blood substitutes. Moreover, using $^{19}$F-MRI is an attractive technique as it provides positive contrast, and the high-resolution anatomical $^1$H clinical images can be registered and superimposed with the highly sensitive $^{19}$F hotspot images to create a precise mapping of where the $^{19}$F-labeled NK cells exist in relation to the patient’s tumor. The ability for repeated imaging over time with $^{19}$F-MRI will also provide a sense of trafficking patterns as well as NK cell persistence after infusion. Longer lived PET isotopes such as $^{89}$Zr have recently been used in combination with more specific cell labeling strategies such as so-called “click chemistry” to enhance signal half-life and specificity with promising results. However, prolonged exposure to ionizing radiation remains problematic for cell-trafficking approaches in the clinic.

Perfluorocarbon $^{19}$F nanoemulsions have gained interest in the past decade as an imaging modality to ex vivo label immune cells and monitor their biodistribution in vivo using MRI. Ahrens et al. were first to initially demonstrate the ability to efficiently label murine DCs ex vivo with perfluoropolyether (PFPE) agents. In their study, they showed that PFPE does not affect DC function in vitro and were able to track DC migration in mice from the foot pad to the popliteal lymph node 6 h post injection. Intravenous injection of PFPE-labeled murine DCs were effectively visualized using an 11.7 Tesla vertical bore MRI and accumulated mainly in the liver and spleen. The same group has subsequently employed $^{19}$F-MRI to track and quantify: autoreactive mouse T cells, antigen specific T cells, monocyte and macrophages in two rat models of renal and cardiac allograft rejection, and PFPE-labeled human primary DCs in immunodeficient mice. Other investigators have also reported on the ability to ex vivo label other potential cell therapies with $^{19}$F, including murine and human neural stem cells, human CD34+ hematopoietic stem cells (HSCs) and mouse bone marrow derived HSCs as well as murine and human mesenchymal stem cells. All of the aforementioned groups have demonstrated that $^{19}$F labeling of immune and non-immune cells does not alter their cell viability, phenotype, proliferation or function in vitro compared to unlabeled cells.

Furthermore, Ahrens et al. were first to prove the feasibility of labeling human immune cells and monitor their migration in mice, although duration of imaging was reported only for 18 h post injection of the human DCs. This has led to usage of $^{19}$F in a phase I clinical trial to monitor an autologous DC vaccine in colorectal patients. Initial results from three patients have recently been published showing the ability to detect human DCs by $^{19}$F-MRI without apparent toxicity. About 10 million DCs were injected intradermally into quadriceps and patients were imaged for about 9.5 min. DCs were detected 4 h post inoculation with a 50% decrease in $^{19}$F signal at the site of injection by 24 h post inoculation in two patients. These data demonstrate the feasibility to visualize human immune cells using $^{19}$F-MRI, however long-term studies in patients are needed to confirm persistence of $^{19}$F signal from the adoptively transferred cells, toxicity and potential false positive signal from death of transferred cells leading to transfer of $^{19}$F signal to other host cells. To our knowledge no report has applied $^{19}$F-MRI to track human NK cells in vivo.

Several other imaging techniques have been used in pre-clinical or clinical scenarios to monitor NK cells in recipients and monitor the tumor response. These techniques include positron emission tomography (PET), single photon emission computed tomography (SPECT), and optical imaging (OI) (bioluminiscence and fluorescence imaging). In vivo studies using immunocompetent mice or xenogeneic mouse models, mouse NK cells or human NK cell lines (NK92) have been labeled with the radionuclides $^{18}$FDG or $^{11}$C-methyl iodide for detection by PET. Allogeneic or autologous human NK cells were also labeled ex vivo with the gamma emitting radioisotope indium-111 oxine ($^{111}$In) for detection by SPECT in patients with renal cell carcinoma and in patients with colon cancer with liver metastasis. In the renal cell carcinoma patients, the NK cells initially accumulated to the lungs, after intravenous injection, and redistributed to the liver and metastatic tumors (lungs and livers) within 24 h. Conversely, in the study with the colon cancer patients, NK cells were only detected within the tumor after intra-arterial delivery of the NK cells. However, the inability to distinguish from cell-bound versus free $^{111}$In might give false positive for the true localization of NK cells.

PET/SPECT imaging provides high sensitivity and specificity, and could be immediately translated to the clinic through the use of FDA-approved radioisotopes ($^{18}$FDG and $^{11}$In). However, several limitations to PET/SPECT still exist. These include mainly ionizing radiation exposure to the patients and fast decay of the tracers ($t_{1/2} = 2.8$ d for $^{11}$In, $t_{1/2} = 109$ min for $^{18}$FDG, and $t_{1/2} = 20$ min for $^{11}$C) which would limit the ability for longitudinal studies to monitor the persistence and biodistribution of the adoptively transferred cells. Moreover, these radionuclide-based methods have low anatomical resolution (PET/SPECT) and have to be combined with other modalities such as CT (CT) or MRI, which increases the complexity of the methodology and the costs. Using optical imaging (OI), NK cells or NK cell lines (NK92) can be detected in vivo after direct ex vivo labeling with organic fluorochromes (i.e., quantum dots,
Cy5.5) \(^{33,35,54}\) or indirectly via transfection of a reporter gene for bioluminescence imaging (BLI) (i.e., luciferase or green fluorescent protein (GFP)). \(^{35,56}\) The advantages of OI methods over MRI or PET/SPECT include cheaper cost of instrumentation, fast imaging, and radiation-free imaging. However, OI is also limited by poor resolution (2–3 mm), lower sensitivity in the case of GFP due to auto-fluorescence and toxicity for certain organic fluorochromes (i.e., quantum dots contain cadmium). Importantly, the main disadvantage to optical methods for cell tracking in vivo is its limited tissue penetration of light (1 mm for fluorescence; 3 mm for bioluminescence), which restricts its application to only small animal imaging and currently there are no available instrumentation for clinical application of optical imaging. \(^{8,9,52,57}\)

Compared to PET or OI, MRI is unique as it allows for higher resolution images (100 μm) regardless of tissue depth, does not use ionizing radiation, and allows for high specificity of signal from the transferred cells. However, it is very difficult to generate the contrast necessary to visualize the transplanted cells due to the high \(^1\)H background signal from water in vivo. Different images acquired before and after the transfer of the labeled cells can improve contrast but are problematic due to variations in the patient’s positioning and due to motion. Additionally, if using gadolinium (Gd\(^{3+}\)), it has to be chelated to limit its toxicity and has finite stability so may be problematic for extended residence times in vivo due to eventual release in tissues as free ion. While MRI offers the most beneficial methodology to track immune cells in vivo, conventional contrast agents used clinically do not allow for quantitative and longitudinal assessment of the transferred cells.

Labeling of NK cells with \(^{19}\)F has been reported to be challenging. \(^{35}\) Our data shows for the first time that \(^{19}\)F labeling of human NK cells is feasible, nontoxic and does not appear to affect viability, cytotoxicity or cytokine secretion by NK cells. We also observed no impact on expression of chemokine or activating receptor involved in NK cell cytotoxicity. Since \(^{19}\)F is passively taken up by NK cells, labeling can be performed in a relatively short period of time (24 to 48 h) at the end of an ex vivo expansion, and does not require extensive manipulation of NK cell cultures. Using subcutaneous and intratumoral injection, we were able to detect infused \(^{19}\)F-labeled NK cells in vivo. In human tumor bearing mice, we are able to show a loss of \(^{19}\)F-signal from the NK cell injection site, which is indicative of the migration of the NK cells from the primary injection site to distant organs and perhaps to the tumor site. Also for the first time, we show that an immune effector cell can be visualized within a tumor, via intratumoral injection, using \(^{19}\)F-MRI. Intratumoral injection of cytokines and ACT has been used previously as an effective route of cancer immunotherapy, \(^{58,59}\) and provides “proof of concept” evidence that NK cells can be imaged within a bulky tumor microenvironment.

All previously published data on \(^{19}\)F-MRI imaging of mouse or human immune cells in vitro or implanted in xenogeneic mice have used MRI scanners with field of strength ranging between 7.0T and 11.7T with the shortest imaging session of 1.5 h and up to 3 h per animal. \(^{14,25-27,30-32}\) Conversely, we used a 4.7T MRI scanner with the total imaging session per animal not exceeding 1 h of scan time. The strength of our MRI is closer to the field strength of MRIs in clinical practice today, making our imaging methodology more relevant for clinical imaging of ACT. \(^{34}\) One obvious limitation of our MRI coil was in the inability to detect \(^{19}\)F-labeled NK cells after intravenous (i.v.) infusion (data not shown), the most common route of therapy. Typically, cells infused i.v. first accumulate in the lungs which has a low water content (and thus low \(^1\)H signal) and is difficult to image with MR due to susceptibility related signal loss via multiple air-tissue interfaces. Improved imaging protocols that use pulse sequences with shorter echo times, \(^{60-63}\) or surface coils that provide high sensitivity in a localized region may improve sensitivity in the lungs to allow imaging of \(^{19}\)F labeled NK cells after i.v. injection.

Concurrent with the promise of \(^{19}\)F MRI for tracking NK cells in vivo, there are several remaining limitations that must be recognized and/or overcome when translating into patients. Field strengths of clinical MRI systems limit detection in vivo to about \(10^5\) cells, which may preclude detection of cells after trafficking to tumors from ancillary delivery intravenously or percutaneously. Nonetheless, optimal field strength, imaging sequence, and scan time needed to obtain sufficient SNR has not yet been fully assessed and could improve sensitivity. In addition, if the \(^{19}\)F-labeled NK cells were to undergo cell death, macrophages or dendritic cells could phagocytose them, also leading to false positive signals. Verification of \(^{19}\)F-labeled NK cells will therefore still need to be done pre-clinically with fluorescent or optical methods until better alternatives are available.

**Methods**

**Animals**

Female NOD.Cg-PrkdcscidIl2rgtm1Wjl/SzJ (NSG) mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) and used between 8–16 weeks of age. All animals were bred and housed in a pathogen-free facility throughout the study. The Animal Care and Use Committee at the University of Wisconsin approved all experimental protocols.

**Reagents and tumor cell lines**

Peripheral blood mononuclear cells (PBMCs) and NK cells were cultured in NK media containing X-VIVO-10 (Lonza, cat#: 04-380Q) supplemented with 10% human AB serum (Corning Cellgro Inc., cat#: 35-060-01), 1% penicillin-streptomycin-glutamine (HyClone, cat#: SV30082.01), and 100 IU/mL recombinant human Interleukin-2 (National Cancer Institute BRB Preclinical Repository). The human chronic myelogenous leukemia cell lines K562 (American Type Culture Collection) and K562-41BBL-IL15-GFP (Waismann Biomanufacturing University of Wisconsin Madison) were maintained in RPMI-1640 (Corning Cellgro Inc., cat#: 15-040-CV) medium supplemented with 10% heat-inactivated FBS (Gibco, cat#: 10437-028) and 1% penicillin-streptomycin-glutamine (HyClone, cat#: SV30082.01) at 37°C in a humidified 5% CO₂ atmosphere. CHLA-20 human neuroblastoma was a gift from Dr Mario Otto (University of Wisconsin), initially obtained from the Children Oncology Group (COG) cell line repository. M21 human melanoma was obtained from the laboratory of Dr Paul Sondel (University of Wisconsin) and gifted by Dr Ralph Reisfeld (La Jolla, CA). Z138
human mantle cell lymphoma was purchased from the American Tissue Culture Collection. CHLA-20, M21 and Z138 cell lines were grown in RPMI-1640 medium containing 10% heat inactivated FBS and 1% penicillin-streptomycin-glutamine, as formulated above, and cultured at 37°C in a humidified 5% CO₂ atmosphere.

**In vivo tumor models and hu14.18-IL-12 immunocytokine**

Tumor cell viability was checked by trypan blue (Thermo Scientific, cat#: SV30084.01) and counted with a hemocytometer prior to in vivo implantation. CHLA-20, Z138 and M21 live cells at 4 x 10⁶ were suspended in 0.1 mL PBS and injected subcutaneously into the right flanks of NSG mice. Once tumor sizes reached on average 8 x 8 cm (diameter x length), human NK cells were labeled with 4 mg/mL ¹⁹F for 24 h, then extra ¹⁹F was washed away from the NK cell cultures and ¹⁹F-labeled NK cells were resuspended in 0.1 mL PBS and injected intratumorally at 1 x 10⁶ or 7 x 10⁶ cells into CHLA-20 or Z138 tumors, respectively.

For mice bearing the M21 tumors (right flank), 10 x 10⁶ ¹⁹F-labeled NK cells were resuspended in 0.1 mL PBS and injected subcutaneously in the left flank opposite to the tumor. For NSG mice bearing M21 tumors, mice were treated intraperitoneal (1 x 10⁶ IU/0.2 mL) with recombinant human IL-2 (National Cancer Institute BRB Preclinical Repository) on days 0 and 7 and treated intratumorally (50 µCi ⁵¹Cr (NEZ030S; Perkin Elmer) per 10⁶ cells and incubated for 60 min at 37°C, then cells were washed to remove extra ⁵¹Cr and target cells were resuspended in media. K562 targets (5 x 10⁵) were added to each well at different effector to target (E:T) ratio and incubated at 37°C for 4 h. The γ-scintillation of supernatant was measured by a γ-counter (Perkin Elmer). Maximum release was determined by adding 100 µL of 1X-Trition X-100 detergent (Sigma-Aldrich, cat#: 9002-93-1) to target cells. Spontaneous release was determined by adding 100 µL of media to 100 µL of target cells. Specific ⁵¹Cr release was calculated as: % lysis = 100% x (Experimental-Spontaneous)/(Maximum-Spontaneous).

**Isolation of NK cells**

Healthy donor peripheral whole blood was obtained through an IRB-approved protocol (2012-0130-CR002). PBMCs were isolated by the buffy coats by density-gradient separation, and the lymphocyte fraction was isolated from PBMCs using Ficoll (Stem Cell Technologies, cat#: 07861). For NK expansion from PBMCs, K562-41BBL-IL15 feeder cells were gamma-irradiated at 100 Gy (JL Shepherd model109 Cesium irradiator) and co-cultured with PBMCs at a 1:1 ratio of feeder cells to PBMCs in NK media. PBMCs and feeder cells were cultured at 37°C in 5% CO₂ on a shaker for 12 d. Fresh media was supplemented on days 4 and 8. On day 12, human NK cells were isolated by negative selection using magnetic cell separation beads (Miltenyi Biotec, cat#: 130-092-657) and sorted using autoMACS® Separator (Miltenyi Biotec Inc., San Diego CA). NK cell purity was determined by flow cytometry staining for anti-CD3 and anti-CD56 and purity was >95% (CD3⁻ CD56⁺).

**¹⁹F labeling and injection of NK cells**

Human NK cells were cultured for 24 or 48 h in a commercially available emulsified PFPE tracer agent CS-ATM-1000 (Celsense, Pittsburgh, PA) in NK media. After 24 or 48 h, NK cells were harvested and washed three times to remove excess PFPE before in vitro and in vivo testing. NK cell viability before and after ¹⁹F labeling was assayed using a standard trypan blue (Thermo Scientific, cat#: SV30084.01) exclusion method. For in vivo studies, ¹⁹F-labeled NK cells were injected into NSG mice subcutaneously or intratumorally.

**NK cell cytotoxicity**

Human NK cell cytotoxic function was determined by a standard 4-h ⁵¹Cr-release assay against the NK cell–sensitive tumor cell line K562 using ¹⁹F-labeled or unlabeled NK cells as effector cells. K562 cells (targets) were labeled with 50 µCi ⁵¹Cr (NEZ030S; Perkin Elmer) per 10⁶ cells and incubated for 5 h at 37°C, then cells were washed to remove extra ⁵¹Cr and target cells were resuspended in media. K562 targets (5 x 10⁵) were added to each well at different effector to target (E:T) ratio and incubated at 37°C for 4 h. The γ-scintillation of supernatant was measured by a γ-counter (Perkin Elmer). Maximum release was determined by adding 100 µL of 1X-Trition X-100 detergent (Sigma-Aldrich, cat#: 9002-93-1) to target cells. Spontaneous release was determined by adding 100 µL of media to 100 µL of target cells. Specific ⁵¹Cr release was calculated as: % lysis = 100% x (Experimental-Spontaneous)/(Maximum-Spontaneous).

**NMR**

To determine labeling efficiency and quantify ¹⁹F uptake by human NK cells, ¹⁹F nuclear magnetic resonance (NMR) spectra were acquired on ¹⁹F-labeled NK cell pellets. Human NK cells (3 x 10⁶ or as indicated in the figure legends) were labeled with ¹⁹F-PFPE (Celsense, CS-ATM-1000), pelleted to discard any ¹⁹F remaining in the culture medium, and the dry cell pellets were lysed in a lysis buffer containing 1% Triton X-100 (Sigma Aldrich, cat#: T6508) diluted in deuterium oxide (Sigma Aldrich, cat#: 7789-20-0). NK cells in lysis buffer were placed in a capillary tube. The added amount of TFA as well as both a chemical shift (−76 ppm) and quantification (three ¹⁹F atoms per TFA molecule) reference for analysis. Samples were transferred into NMR vials and further diluted with D₂O such that the cell lystate solution fit the entire detection region of the NMR coil. One-dimensional (1D) ¹⁹F NMR spectra were obtained on the NK cells using a 9.0 s recycle delay (TR), 90° flip angle, and 128 averages with the primary ¹⁹F NMR peak from the NK cells identified around −91 ppm. All spectra were acquired using a 9.4T vertical bore Varian Unity-Inova NMR spectrometer (Agilent, Santa Clara, CA, USA). The following equation was used to determine the ¹⁹F-labeling density,

\[ F_L = \frac{3 \times I_L \times M_R \times N_A}{I_R \times N_L} \quad (1) \]

where \( F_L \) is the number of ¹⁹F atoms per cell, \( I_L \) is the integrated area of major peak of the NK cell pellet, \( M_R \) is moles of TFA reference added, \( N_A \) is Avogadro’s number, \( I_R \) is the integrated area under the TFA reference peak, and \( N_L \) is the number of
cells in pellet. The average $F_c$ was taken as the mean from three separate samples and its uncertainty ($\sigma_{F_c}$) was estimated as the standard deviation. NMR peak integration was performed in VNMR 6.1C (Agilent, CA, USA) with plotting performed in MATLAB R2014b (The Mathworks Inc. MA, USA).

**In vivo MRI**

Prior to MRI, mice were anesthetized with 1 mg ketamine (Ketaset, NDC#: 57319-542-02) and 0.1 mg xylazine (LOYD Inc., NADA#: 139-236) per 10 g body weight, monitored with a respiration pad, and maintained at 36°C using a warm-air blower. An external phantom vial containing PFPE with known $^{19}$F spin density ($\rho_R$) of $2.3 \times 10^{16}$ $^{19}$F atoms/mm$^3$ was placed in the field-of-view (FOV) and used as a $^{19}$F reference during image acquisition. MRI was performed on a 4.7 T small animal MRI system (Agilent Technologies, Santa Clara, CA) using a volume quadrature $^{19}$F coil tunable to $^{19}$F (187.9 MHz) and to $^1$H (200 MHz). Multiple coronal $^{19}$F 2 mm slice images were acquired using a multi-slice spin echo acquisition with 2000/9.0 ms TR/TE, 16 echoes, 16 kHz receiver bandwidth, 72 $\times$ 36 mm FOV, 64 $\times$ 32 matrix size, 1.1 $\times$ 1.1 mm$^2$ in-plane resolution, and 40 averages resulting in a 42.6 min total scan time for $^{19}$F imaging. The same coil was used to acquire anatomical $^1$H images using either a $T_1$-weighted gradient echo acquisition with an 80.4/3.4 ms TR/TE, 20° flip angle, and 0.28 $\times$ 0.28 mm$^2$ in-plane resolution or a $T_2$-weighted fast spin echo with a 3500/16.5 ms TR/TE, 8 echo train length and 0.28 $\times$ 0.28 mm$^2$ in-plane resolution. The FOV and slice thickness (2 mm) were identical for both $^{19}$F and $^1$H images to allow for easy co-registration between nuclei. One mouse bearing the human neuroblastoma CHLA-20, one mouse bearing the human mantle cell lymphoma Z138 and two mice bearing the human melanoma M21 were imaged by MRI for quantification of the number of $^{19}$F-labeled NK cells and their localization in vivo at different time points (indicated in the figure legends for each tumor model).

**Quantitative analysis of $^{19}$F signal in vivo**

All image analysis was performed in MATLAB R2014b (Mathworks; Natick, MA). Quantitation of the number of cells based on $^{19}$F signal was performed similar to the previously published algorithm. Briefly, low-SNR magnitude image voxels were corrected for their Rician distribution by generating a look-up table between measured and expected signal intensity. Using a region-of-interest (ROI) drawn in the noise/background, the standard deviation of either the real or imaginary signal of the complex $^{19}$F MR data was calculated ($\sigma_N$) on a per-slice basis. This value was used to generate the look-up table and voxels with signal below a threshold of 8 $\times$ $\sigma_N$ were corrected to lower signal values. Signal levels above 8 $\sigma_N$ were not corrected as they approach a Gaussian distribution. Further, a minimum signal threshold of 5 $\sigma_N$ was applied to select voxels with $^{19}$F signal above the noise. For visualization, $^{19}$F magnitude images were interpolated to match the $^1$H image matrix size. $^{19}$F images were overlaid on $^1$H images for anatomical positioning of $^{19}$F signal with the above signal threshold applied. All the voxels with $^{19}$F signal above the signal threshold ($S_V$) were summed for all slices. The average signal in the reference vial was measured with a drawn ROI ($\overline{S}_R$). The spin density of the reference ($\rho_R$) was normalized to the imaging voxel size in units of $^{19}$F atoms per voxel. The apparent number of cells ($N_c$) in the MR image could then be computed using the previously determined $F_c$ (see Equation 1 under NMR methods) and Equation 2 below.

$$N_c = \sum (S_V) \times \frac{1}{F_c} \times \frac{\rho_R}{\overline{S}_R}$$  \hspace{1cm} (2)

To determine the uncertainty in $N_c$, ($\sigma_{N_c}$), Equation 3 below was used.

$$\left(\frac{\sigma_{N_c}}{N_c}\right)^2 = \left(\frac{\sigma_{S_V}}{S_V}\right)^2 + \left(\frac{\sigma_{F_c}}{F_c}\right)^2 + \left(\frac{\sigma_{\rho_R}}{\overline{S}_R}\right)^2$$  \hspace{1cm} (3)

The uncertainty in the $^{19}$F reference mean signal ($\sigma_{S_R}$) was estimated as the standard deviation of the ROI drawn on the reference. The uncertainty in the summed $^{19}$F voxel signal ($\sigma_{S_V}$) was estimated by Equation 4 below:

$$\sigma_{S_V} = \sigma_N \times \sqrt{2 \times n}$$  \hspace{1cm} (4)

where $\sigma_N$ is the standard deviation of the noise in the MR image and $n$ is the number of voxels identified as having $^{19}$F signal above the noise threshold. No uncertainty was assumed in the spin density in the $^{19}$F reference ($\sigma_{\rho_R} = 0$).

**Flow cytometry**

Human NK cells were cultured for 24 h with different concentrations of $^{19}$F PFPE or cultured without label as a control. After labeling, 1 $\times$ 10$^6$ NK cells were incubated with Fe block clone 3G8 (BD Pharmingen, cat#: 564220) and stained at 4°C for 20 min with anti-human antibodies including FITC-CD3 (OKT3, cat#: 317306), PE-IgG1k (MOPC-21, cat#: 400114), PE-NKp44 (P44-8, cat#: 325108), PE-DNAM-1 (11A8, cat#: 338306), PeCy7-NKp2D (1D11, cat#: 320812), PeCy7-IgG1k (MOPC-21, cat#: 400126), PeCy7-NKp46 (9E2, cat#: 331916), AF647-NKp30 (P30-15, cat#: 325212), AF647-IgG1k (MOPC-21, cat#: 400130 and 400136), AF647-IFNy (4S.B3, cat#: 502516), AF647-Granzyme B (GB11, cat#: 515405), APC-CX3CR1 (2A9-1, cat#: 341609), APC-CXCR4 (12G5, cat#: 306510), BV421-CD56 (HCD56, cat#: 341609), BV510-CX3CR1 (2A9-1, cat#: 345405), APC-CXCR4 (12G5, cat#: 400114), and PE-NKp44 (P44-8, cat#: 325108). The cytometry data was acquired on a MACSQuant analyzer 10 (Miltenyi Biotec Inc., San Diego, CA) and fcs files were converted to fcs files using The MACSQuantify™ Software. List-mode data were analyzed using FlowJo software (TreeStar).
ELISA

Human IFNγ levels in supernatants from unlabelled human NK cells or 19F-labeled NK cells were quantified using Legend Max ELISA kit with pre-coated plates with human IFNγ according to manufacturer’s instructions (BioLegend, Inc., cat#: 430107). IFNγ cytokine concentration was extrapolated relative to a standard curve created by serial dilution of human IFNγ standards run in parallel. ELISA plates were at 450 nm on a VERSAmax Tunable Plate Reader (Molecular Devices, Sunnyvale, CA) and data were collected using SOFTmax PRO software (Molecular Devices, Sunnyvale, CA).

Statistical analysis

Statistics were performed using GraphPad Prism version 6.0 for the Macintosh OS (GraphPad Software, San Diego, CA). Data were expressed as mean ± SEM. For analysis of three or more groups, the non-parametric ANOVA test was performed with the Bonferroni or Sidak’s multiple comparisons post-test. Analysis of differences between two normally distributed test groups was performed using the Student’s t-test. Welch’s correction was applied to Student’s t-test data sets with significant differences in variance. A p value less than 0.05 was considered statistically significant.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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