Monitoring of Natural Killer Cell Immunotherapy Using Noninvasive Imaging Modalities

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Abstract
Cancer immunotherapies can be guided by cellular imaging techniques, which can identify the presence or absence of immune cell accumulation in the tumor tissue in vivo and in real time. This review summarizes various new and evolving imaging techniques employed for tracking and monitoring of adoptive natural killer cell immunotherapies, the design of related clinical trials, and ultimately, assessment of these therapies in clinical practice.

Introduction
Adoptive transfer of in vitro activated or genetically modified natural killer (NK) cells represents an evolving approach of cancer immunotherapy. The non–major histocompatibility complex (MHC)-restricted killing of tumor cells by NK cells provides advantages over T cells and makes the use of NK cells appealing as potential effectors for immunotherapy. The capability of ex vivo expanded NK cells of homing to tumors, and retaining their cytotoxicity once transferred from the in vitro to in vivo tumor microenvironment, is frequently questioned by oncologists (1). Strategies aimed at maximizing NK-cell trafficking to tumors or secondary lymphoid organs are being employed (e.g., NK-cell combination with bortezomib, or with lympho-depleting chemotherapy of high-dose Cytoxan and fludarabine) to exploit the full therapeutic potential of NK-cell–based immunotherapy for cancer (1). Major obstacles in developing new immunotherapeutic approaches include the lack of a noninvasive tool for in vivo monitoring of immune effector cells, quantifying effector-to-target (NK cell-to-tumor) ratios, and cytotoxicity outcomes (2). To date, the identification of responders and nonresponders to NK-cell–based immunotherapies relies on a decline in tumor markers or a reduction in tumor size, weeks or months after initiation of treatment (3–6). With imaging techniques, the presence, quantity, and distribution of NK cells in target tumors can be monitored noninvasively, instantly, and in real-time (3–10). The acquired imaging data may serve as a surrogate marker for tumor response. Thus, NK-cell imaging could immediately and positively affect preclinical assessments of new combination and/or adjuvant immunotherapies, the design of related clinical trials, and ultimately, assessment of these therapies in clinical practice.

Labeling of NK Cells with Tracers or Contrast Agents for In vivo Tracking
NK cells can be tracked with radioisotope imaging techniques: positron emission tomography (PET) and single photon emission computed tomography (SPECT); magnetic resonance (MR) imaging techniques; and optical imaging (OI) and fluorescence and bioluminescent imaging (BLI). For tracking with PET, NK cells were labeled with positron-emitting radionuclides:¹⁸F and¹¹C (11, 12). NK cells were labeled with γ-photon-emitting radioisotope¹¹In for SPECT imaging (3–5). Both PET and SPECT provide high sensitivity (single cell) for NK-cell depiction, high target-to-background contrast, in vivo quantification of labeled cells, immediate clinical applicability because of U.S. Food and Drug Administration (FDA)–approved labels, and established whole-body imaging techniques (3–5, 10, 11). Limitations include high expense; low resolution when used without computed tomography (CT); 1 to 2 mm; radiation exposure; and tracer decay, limiting follow-up studies to 2 to 3 hours with¹⁸FDG or 4 to 7 days for¹¹In (3–5, 10). The low resolution of PET and SPECT images can be improved by integration with high-resolution images delivered by CT. This modality allows for better correlation of PET-SPECT signals to anatomic structures visualized with CT. The given resolution for the CT part of hybrid PET-CT or SPECT techniques is 200 to 400 μm. Of note, different SPECT radionuclides emit positrons at different energy levels, which can be distinguished by the γ-cameras. This method can be used to label two different cell populations (e.g., NK cells and T cells) with different SPECT isotopes and track them at once in the same subject. Conversely, all PET radioisotopes emit two γ-photons of the same energy (511 keV), thereby omitting parallel tracking of different cell types (3–5, 10, 13).
NK-cell tracking with OI requires either of the following methods: (A) labeling of target cells with an exogenous fluorescent dye for fluorescence reflectance imaging (Fig. 1; ref. 9) or (B) transfection of target cells with a gene encoding the...
synthesis of either (B1) a fluorescent protein [e.g., green fluorescent protein (GFP)], detectable with fluorescence imaging, or (B2) luciferase, detectable with BLI (9). Methods (B1) and (B2) offer the distinct advantage of providing information about the viability of the labeled cells because the fluorescence or bioluminescence signal disappears when the labeled cell undergoes apoptosis (8, 9, 14). Only method (A) is, in principle, clinically applicable, when using the FDA-approved label indocyanine green (ICG). OI offers inexpensive, fast, and radiation-free in vivo monitoring of NK cells with high sensitivity and low background noise [especially with near-infrared (NIR) fluorophores; refs. 9, 14]. Limitations include limited tissue penetration (1 cm with fluorescence imaging and 3 cm with bioluminescence imaging), low resolution (2-3 mm), two-dimension projection technique, and underdeveloped human imaging devices (8, 9). Some of these obstacles are

Figure 1. A, NK cells are labeled with a radiotracer (111In or 18FDG), contrast agent (ferumoxides or DiD), or transfected with DNA encoding a luciferase reporter. B, representative examples depicting NK-cell tumor accumulation with various imaging modalities. Sections through mice represent axial imaging modalities: Clockwise, ex vivo PET images obtained 2 hours after Her2neu-targeted NK-cell injection show an increased signal in the tumor, whereas no signal is noted in the tumor after injection of parental cells. SPECT images show tumor accumulation of NK cells in liver metastases (arrow), after intra-arterial injections in a clinical model. Reproduced with permission from Matera and colleagues (5). BLI shows accumulation and persistence of NK cells in tumor xenografts from days 0 to 12. Reproduced with permission from Edinger and colleagues (8). Axial MR images before and 24 hours after injection of ferumoxide-labeled NK cells: Postinjection MR image shows a decline in signal intensity due to NK-cell accumulation in the tumor (arrow).
being addressed with the development of new handheld endoscopic and tomographic imaging systems, such as fluorescence molecular tomography (FMT), FMT-CT, and FMT-MRI (8, 9). A prototype clinical OI scanner is currently being investigated for breast cancer imaging in patients (15).

MR imaging detects relativity of protons within a magnetic field as a signal. MR contrast agents alter proton relaxivity leading to signal enhancement on MR images without being imaged directly. In order to track NK cells with MR imaging, they are typically labeled with iron-oxide nanoparticles producing a strong negative (dark) enhancement on T2-weighted images (Fig. 1). Several iron-oxide nanoparticles are suitable for clinical use, such as ferumoxides (FDA-approved in the United States, Europe, and Japan for liver imaging), ferumoxylt (FDA-approved for treating iron deficiency), and ferucarbotran (approved for liver imaging in Europe and Japan; refs. 7, 16, 17). MR imaging offers many advantages, such as readily available clinical translation, high resolution (100 μm in plane), high soft-tissue contrast, absent radiation exposure, and longer persistence of the signal (2-4 weeks; refs. 14, 18). Limitations of MR cell-tracking techniques include high costs, long scan times, and limited sensitivity (detects 40 to 50 cells; refs. 14, 18). Additionally, the MR signal is not directly related to contrast agent or cell concentrations, making it difficult to quantify target cells in vivo (14, 18).

**Monitoring NK-Cell Tumor Accumulation: Preclinical In vivo Studies**

Radiotracers have been extensively used for tracking leukocytes to inflammations and tumors. PET using the \( ^{11}C \) methyl iodide label [half-life \( t_{1/2} = 20 \) minutes] was used to track activated NK cells and nonactivated lymphocytes to fibrosarcoma xenografts (Fig. 1; Supplementary Table S1; ref. 11). At 1 hour postinjection, 4 to 30% of the activated NK cells had accumulated in the tumor compared with 3 to 4% of nonactivated cells. This study pioneered initial applications of NK-cell tracking to target tumors with PET (11). As a translational approach, FDA-approved \(^{18}F\)FDG was used to label genetically engineered Her2neu-directed NK cells and track them to Her2neu-expressing breast sarcoma xenografts (10). Ex vivo autoradiography scans showed 2.9 times higher radioactivity in tumors treated with genetically engineered NK cells than parental NK cells (Supplementary Table S1). This study furnished proof of concept for NK-cell tracking with a widely available, clinically applicable PET tracer (10). However, the short half-life of \(^{18}F\)FDG (109 minutes) limits in vivo monitoring of \(^{18}F\)FDG-labeled NK cells to 2 to 3 hours (10). Other PET tracers with longer half-lives [e.g., \(^{12}I\) \( t_{1/2} = 4.18 \) days, \(^{89}Zr\) \( t_{1/2} = 3.27 \) days, and \(^{86}Y\) \( t_{1/2} = 2.67 \) days] have been used to track labeled antibodies (bevacizumab, trastuzumab), and are yet to be exploited for cell tracking (19).

BLI has been used to track GFP-luc transfected NK cells to A-20 lymphoma xenografts (Fig. 1). Because a genetic modification led to the luminescent signal, it did not fade with cell proliferation and allowed for long-term cell tracking after NK-cell administration. After injection of \( 5 \times 10^6 \) NK cells (25% GFP+) in these mice, the tumors completely regressed at day 12 and remained undetectable at 6-month follow-up (Supplementary Table S1; ref. 8). Positive luminescence established the viability of the labeled NK cells (8). Limitations of BLI include relatively low transduction efficiency of retroviral vectors for primary lymphocyte populations and the risk of gene silencing (8). Additionally, some reporter genes could become immunogenic, as described for GFP (8, 14). The use of lentiviral vector systems and the generation of transgenic animals with constitutive or inducible expression of bioluminescent reporter genes are important steps in overcoming these limitations (8). The necessary genetic modification for obtaining bioluminescent cells limits this technique to experimental applications.

Fluorescence reflectance imaging of NK cells labeled with exogenous fluorescent markers has been exploited in experimental and clinical contexts. The NIR dyes, for example, DiD (1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine), DiO (3,3-dilinoleylxycarbocyanine), DiI (1,1'-dioctadecyl-3,3,3,3'-tetramethylindocarbocyanine perchlorate), or VT680 ( VivoTag 680) provide quick, inexpensive, and uncomplicated (simple incubation for few minutes) cell labeling for experimental applications. Other fluorescent labels for cell tracking have been reviewed by Sutton and colleagues (14). DiD labels remain stable for several weeks, can be depicted with both OI and fluorescence microscopy, and have been applied to track tumor-targeted NK cells to prostate cancer xenografts (9). This procedure allowed for close observations of the in vivo distribution of systemically injected NK cells (Fig. 1; Supplementary Table S1; ref. 9). ICG, which is FDA-approved for measuring tissue blood volumes, cardiac output, hepatic function, and tumor perfusion (12), is currently being investigated in our laboratory with the goal to develop clinically applicable optical NK-cell tracking techniques.

Lim and colleagues provided proof of concept of cell tracking with quantum dots (QD) by labeling NK92MI cells with anti-CD56 antibody-coated QD705, and tracking the labeled cells up to 12 days after intratumoral injections with OI (Supplementary Table S1). The authors found a decreased size of tumors treated with NK92MI cells compared with controls (20). Labeling with QDs provides high quantum yield, high sensitivity, stable cell labeling for months, and a very narrow excitation and emission spectrum. This labeling allows for “multiplexing,” which is simultaneous tracking of two or more cell populations, labeled with QDs of different colors, in the same animal (20). QD labeling may be the most well-suited imaging technique for tracking different cell populations at once. Although currently available compounds are not clinically applicable because of toxic cadmium cores or other nondegradable components (21), cadmium-free or biodegradable QDs are currently being developed.

MR imaging has been exploited sparsely for NK-cell tracking, probably because of its high cost. However, MR imaging would be immediately clinically applicable and, thus, deserves increased attention as clinical trials evolve. Our group used MR imaging to monitor NK-cell-based immunotherapies for...
treatment of breast and prostate tumors (Fig. 1; Supplementary Table S1); we monitored the accumulation of ferumoxide-labeled HER2-neu-targeted NK cells in HER2-neu-expressing breast sarcomas, and the accumulation of ferumoxide-labeled epithelial cell adhesion molecule (EpCAM)-targeted NK cells in EpCAM-expressing prostate cancers (Supplementary Table S1; ref. 7). In addition to confirming NK-cell tumor accumulation, MR imaging provided additional details about the distribution of the labeled NK cells within the tumor tissue (7).

Clinical In vivo Applications

Clinically established SPECT techniques have been applied to evaluate the in vivo distribution of systemically injected 111In-labeled NK cells in patients (3–5). Matera and colleagues deduced the target tumor accumulation of NK cells after intra-arterial and intravenous routes of NK-cell administration (Fig. 1; Supplementary Table 2). Signal within the tumor corresponding to delivery of cells was noted only after intra-arterial, and not intravenous, injections (5). Conversely, other authors found that intravenously injected 111In-labeled NK cells initially accumulated in the lungs, then redistributed to the liver, spleen, and bone marrow, and finally accumulated in metastases in lungs and liver 24 hours after injection (Supplementary Table S2; refs. 3, 4). 111In-labeled NK cells could be tracked up to 6 days (t1/2 = 2.8 days; refs. 3, 4). This finding is in accordance with the patterns noted in our laboratory with various imaging modalities and proves that NK cells do reach the tumors and are not immunologically destroyed in circulation (3, 4). Thus, SPECT provides an immediate clinically applicable tool to monitor and tailor immunotherapy protocols with regard to the best route, quantity, and timing of immune-cell administrations.

MR imaging and OI may offer less invasive, radiation-free alternatives, and are ready for monitoring of NK-cell–based clinical trials. Proof of concept for MR imaging has been provided for dendritic cells in patients with melanoma: Ferumoxides and 111In-labeled dendritic cells have been tracked from subcutaneous tissue injections to local lymph nodes using MR imaging, providing congruent information to simultaneously obtained SPECT scans (6). MR imaging allowed better assessment of the accuracy of dendritic cell delivery and cell migration patterns (6).

Future Directions

NK-cell immunotherapy has advantages over other immunotherapy approaches in being non-MHC-restricted, nonimmunogenic, and highly cytotoxic. NK-cell–based strategies promise greatest benefit when used in the setting of minimal residual disease or as an adjunct to other immunome-based therapies. Noninvasive imaging techniques allow for an in vivo evaluation of the accumulation, distribution, and quantity of NK cells within the tumor parenchyma as early as 2 to 4 hours after systemic administration (9, 10). This evaluation may help to understand the in vivo kinetics of parental and retargeted NK cells and facilitate the development of successful and efficacious NK-cell therapies against chemotherapy-insensitive cancers. None of the described labels have been noted to interfere with NK-cell function (7, 9, 10). The best-suited imaging modality for tracking NK cell therapies depends on the desired sensitivity, follow-up interval, and radiation tolerance, as well as available imaging technologies. Of the modalities described, PET and MR imaging combine clinically important attributes of high sensitivity (PET), high resolution (MR), and established imaging protocols for tumor imaging in patients. Thus, emerging developments of hybrid PET–MR techniques may represent the most promising approach for future clinical applications. OI and PET imaging provide for earlier, though short, follow-ups. If rapid image acquisition is required, as in biokinetic studies, these modalities are most suitable. Given the longer stability of ferumoxide labels, MR imaging can achieve long-term follow-up. Applying imaging-based surrogate markers for NK-cell tumor accumulation and tumor response would have a major impact on health care economics by avoiding ineffective treatment of patients who are unknowingly therapy resistant. Because clinical trials of new immunotherapies are expensive and take years to complete, the immediate value and impact of the described imaging techniques are apparent. NK-cell tracking techniques could be, in principle, readily applied in a clinical setting, hold the potential to improve therapy management, and, ultimately, may provide a new tool to improve long-term cancer regression and patient survival.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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