Noninvasive Tracking of Natural Killer Cells Using Gold Nanoparticles

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ABSTRACT: Natural killer (NK)-cell-based immunotherapy is emerging as an attractive approach for cancer treatment. However, to facilitate and expedite clinical implementation, important questions must be answered regarding the in vivo functionality and trafficking patterns of the transferred cells. We have recently developed a noninvasive cell-tracking technique, based on gold nanoparticles (GNPs) as cell-labeling and contrast agents for whole-body computed tomography (CT) imaging. Herein, we report the implementation of this technique for longitudinal and quantitative tracking of NK cell kinetics, the migration and biodistribution in tumor-bearing mice. NK cells were successfully labeled with GNPs, without impairing their biological function, as assessed both in vitro, by cytokine release and cytotoxicity assays, and in vivo, using a xenograft model of human tumors. Using CT, we longitudinally tracked the migration of intravenously injected NK cells and observed an accumulation of effector cell clusters at the tumor site, up to 72 h. Fluorescence imaging of the cells over time correlated with ex vivo quantitative analysis of gold content in the tumor, validating the accuracy and reliability of our technique. Our cell-tracking approach thus offers a valuable tool for preclinical studies, as well as for clinical applications, to elucidate the fate of NK cells and promote the implementation of NK-cell-based immunotherapy.

INTRODUCTION

Cellular immunotherapy is a rapidly expanding paradigm with immense potential for effective cancer treatment. Several immune cell types play important roles in antitumor response, including T cells, dendritic cells, and natural killer (NK) cells. The latter are especially appealing for cell-based immunotherapy due to their intrinsic capacity to recognize and target tumor cells often bypassing HLA restriction, their rapid killing ability, and better safety, as compared to T-cell-based therapy. A large body of recent preclinical and clinical research makes use of the functional effector NK cell line NK92, the only allogeneic cell line currently used in clinical trials. This cell line, isolated in the early ‘90s, was demonstrated to be functional and has several technical advantages over the use of donor-derived peripheral NK cells. Importantly, it displays the ability to detect and eliminate tumor cells and secrete antitumor cytokines such as TNF-α and IFN-γ. NK92 cells, both unmodified and genetically engineered, have garnered success in allogeneic settings in phase I clinical trials, presenting a low-toxicity burden and almost no side effects, as observed in melanoma and renal carcinoma patients. These findings confirm the potential of NK92 cells as a convenient and universal alternative cell platform for immunotherapy.

Despite the recent progress in NK-cell-based immunotherapy, critical issues remain to be defined, including optimal cell dosage, trafficking and migration patterns, survival, and final fate of NK cells post-transplantation. This lack of information is a major obstacle that hinders the more rapid progress of NK-cell-based therapy and prevents it from reaching its full potential. Addressing these challenges requires a reliable, noninvasive, and real-time cell-tracking technique that can continuously evaluate the kinetics, biodistribution, and functionality of NK cells post-injection.

To date, NK cell tracking has been achieved mainly by optical imaging techniques, that is, fluorescence or bioluminescence methods, which are limited by low tissue penetration, or via nuclear-based tracking systems, which use short-lived radionuclide imaging agents. While nanoparticle-based magnetic resonance imaging may offer advantages over NK cell tracking due to its high sensitivity, the contrast generated by nanoparticles can interfere with imaging of the surrounding tissues.

Recently, we developed a novel methodology for longitudinal and quantitative in vivo cell tracking, based on the combination of computed tomography (CT) as an imaging modality and gold nanoparticles (GNPs) as cell-labeling...
agents. CT is one of the most widely used imaging techniques in the clinic, due to its cost-effectiveness, high spatial resolution, short scan time, and ease of imaging processes. Moreover, GNPs have unique physical, chemical, and biological properties, making them attractive contrast agents for CT imaging. In recent years, GNPs have attracted broad interest as in vivo tracking agents, due to their inert and nontoxic nature.

Herein, we developed a sensitive NK-cell-tracking approach based on GNP labeling for precise CT imaging (Figure 1A). First, we established a protocol for efficient NK92 cell-labeling with GNPs, while maintaining NK92 cell functions and viability. Labeled cells were longitudinally tracked in tumor-bearing mice using CT, up to 72 h post-intravenous (IV) injection. The cells mediated tumor growth inhibition independent of GNP labeling, demonstrating the safety of our approach. Overall, this work demonstrates a novel ability to longitudinally visualize clusters of NK cells as they accumulate at the tumor site.

**RESULTS AND DISCUSSION**

*In Vitro Characterization and Functionality of GNP-Labeled NK92 Cells.* GNPs were synthesized and coated with polyethylene glycol (PEG) and D-((β)-glucosamine, as we have recently shown that glucose coating increases the cellular uptake of GNPs. The particles were characterized using transmission electron microscopy (TEM) and UV–vis spectroscopy (Figure 1B,C) and displayed an average size of 20 nm with an absorption peak at 525 nm.

Next, NK cells were incubated with 0.75 mg/mL GNPs for 1 h, as we have previously shown that these conditions allow efficient labeling and optimal biological activity in other immune cell types. The NK cell-uptake efficiency was quantitatively analyzed using flame atomic absorption spectroscopy (FAAS), showing an average uptake of 130 pg Au/cell, which corresponds to 1.6 × 10⁶ particles per single cell. To verify that GNP-labeled NK cells preserve the therapeutic potential, the antitumor activity against specific target cells was examined and cell cycle analysis was performed. GNP-labeled NK92 cells were co-cultured with either the K562 human erythroleukemia cell line (efficiently recognized as a positive target) or the 888 human melanoma cell line (as a negative control) for 18 h. We found that the GNP-loaded NK92 cells secreted IFN-γ and TNF-α at levels similar to those of the nonlabeled NK-92 cells (825 pg/mL IFN-γ and 415 pg/mL TNF-α secretion in GNP-loaded NK92 cells vs 1035 pg/mL IFN-γ and 519 pg/mL TNF-α in controls, p ≥ 0.706) (Figure 2A,B). No significant changes in cytokine secretion levels were noted in co-cultures with the control 888 cell line (p ≥ 0.56, Figure 2A,B).

Likewise, GNP-labeled NK92 cells showed tumor-cell-killing abilities similar to those of nonlabeled NK92 cells (p = 0.997, Figure 2C). Additionally, cell cycle analysis showed no
significant difference in the viability of NK92-GNP loaded cells, as compared to nonlabeled cells (Figure 2D).

These findings demonstrate that GNP-labeled NK92 cells can specifically identify target cells and maintain their cytokine secretion and antitumor functions.

**CT Imaging of NK92 Gold-Labeled Cells in Tumor-Bearing Mice.** We next examined the efficacy of GNP labeling of NK92 cells for longitudinal in vivo tracking and imaging with CT. Mice were subcutaneously injected with either K562 positive target cells or 888 negative target control cells, and after tumor establishment, they received an intravenous injection of either GNP-labeled NK92 cells, nonlabeled NK92 cells, or saline (four mice per group). CT imaging was conducted at 24 h, 48 h, and 72 h post-injection (see the Supporting Information, Figs. S1−S5). Two-dimensional (2D) and three-dimensional (3D) CT imaging revealed that the GNP-labeled NK92 cells accumulated in K562 tumors and formed clusters at the tumor site over time (Figure 3; representative images at 48 and 72 h post-injection are shown). As expected, no signal was detected at the tumor site for the nonlabeled NK92 cells, confirming that the cells could not be visualized without GNP labeling. Furthermore, no signal was detected at the control 888 tumor site, showing that the GNP-labeled NK cells did not accumulate in the control tumors.

**Validation of Cell Tracking by Fluorescence Imaging.** To further characterize and validate our in vivo tracking system, NK92 cells labeled with a red fluorescent dye (CellTracker Deep Red) were intravenously injected into K562 or 888 tumor-bearing mice, visualized with whole-body fluorescence imaging, and compared to the GNP-based technique, as measured by inductively coupled plasma mass spectrometry (ICP), at 24, 48, and 72 h post-injection. Fluorescence imaging showed an accumulation of NK cells at the K562 tumor site over a period of 3 days (Figure 4A), which matched with the CT imaging findings. Additionally, we found that the
fluorescent signal intensity quantified at the tumor correlated with gold accumulation measured in the tumor over time, showing the same trend of increase with a maximum signal reached at 72 h post-injection (Figure 4B,C). It should be noted that the reduced sensitivity of FLI did not enable cell detection at 24 h.

Figure 4. (A) Fluorescence imaging of NK92 cell accumulation at target tumor vs nontarget tumor over time: fluorescence imaging of NK cells in tumor-bearing mice (K562 or 888 cell lines) was conducted over time using the Maestro II in vivo imaging system. The tumor area is demarcated by a circle; NK cells can be seen in red within the K562 tumor. (B,C) Comparison of quantitative ICP and fluorescent signals at the tumor site over time: maximum fluorescence intensity (MFI) quantified at the tumor site over time. Average intensity over the tumor surface area was calculated using ImageJ software (B). Gold quantified in tumor from 24 to 72 h. Average mg Au/gr tissue measured by ICP (C). Both GNP and fluorescence measurements show the same trend of quantitative increase up to 72 h post-injection. The low sensitivity of FLI did not enable cell detection at 24 h.

In Vivo Tumor Regression Mediated by Gold-Labeled NK92 Cells. We next validated if GNP-labeled NK92 cells retained their antitumor therapeutic effect in vivo. The tumor growth rate was evaluated in K562 tumor-bearing mice after intravenous administration of either GNP-labeled or nonlabeled NK92 cells or saline. Both GNP-labeled and nonlabeled NK cells significantly inhibited tumor growth, as compared to saline-treated controls (Figure 5A, p < 0.01 using Student’s t-test).

Figure 5. Therapeutic efficacy and tumor infiltration of GNP-labeled NK92 cells. K562 tumor-bearing mice received an IV injection of either GNP-labeled [NK92(+GNP)] or nonlabeled NK92 [NK92(−)] cells. (A) Labeled and nonlabeled NK92 cells show similar inhibition of tumor growth; the difference between the two NK92-treated groups and saline-treated controls is statistically significant (p = 0.0034). (B) Tumor infiltration levels of GNP-labeled NK92 cells are similar to those in the nonlabeled NK92-treated group, on day 11 (p = 0.396). Results are presented as a percentage of total single-cell suspension and mean ± SEM.
At the end of the experiment (day 11), tumors were excised and analyzed by flow cytometry for CD56 expression (an NK cell marker), to assess the level of tumor-infiltrating NK92 cells. A similar percentage of GNP-labeled or nonlabeled NK92 cells was found within tumors (Figure 5B; 25.3 and 20% NK cells, respectively; \( p = 0.396 \)).

These findings demonstrate that GNP-labeled and nonlabeled NK92 cell traffic and infiltration of the tumor were at similar levels and mediated a similar antitumor effect. Taken together, these results confirm that GNPs do not impair the therapeutic effect of NK92 cells in vivo.

**Biodistribution of GNP-Labeled NK92 Cells.** To determine the biodistribution of NK92 cells following IV injection, mice treated with GNP-loaded NK cells were sacrificed at 24, 48, and 72 h after injection, and their organs were excised and analyzed for gold content using FAAS. Significant gold uptake was found in clearance organs, namely, the spleen and the liver, as well as in the lungs. Lung accumulation gradually decreased over time (Figure 6). No gold was detected in the blood and kidney 24 h post-injection.

**Figure 6.** Biodistribution of GNP-labeled NK92 cells in mice. Gold concentrations in major organs 24, 48, and 72 h post-IV injection of GNP-labeled NK92 cells, as quantitatively measured by FAAS. No significant amount of gold was detected in the kidney and blood.

### DISCUSSION

Developing sensitive techniques for non-invasive in vivo cell tracking is essential for elucidating mechanisms underlying adoptive transfer treatments. In this study, we utilized GNPs as CT-imaging contrast agents for tracking NK cells in vivo. Our approach provided important information on NK92 cell trafficking kinetics, biodistribution, and fate.

Reliable nanoparticle-based cell tracking requires several key parameters. The first is efficient cell labeling for successful imaging. Here, we achieved loading of a sufficient number of particles into each cell and thereby attained satisfactory contrast. Importantly, the cells maintained their biological function and viability and showed a maximal therapeutic effect, as demonstrated both in vitro and in vivo. The second parameter is the accurate analysis and interpretation of the obtained images; identifying small clusters of cells and differentiating them from the surrounding soft tissue is a challenging task. Our GNP-based approach overcame this challenge by achieving a distinct CT signal that enabled discerning the accumulation of cells at the tumor site over 72 h. Finally, as longitudinal cell tracking involves imaging days after treatment, it is important to validate whether the tracked nanoparticles indeed remained within the cells over time.

Herein, the longitudinal NK92 cell tumor accumulation observed by CT up to 72 h was found to be comparable with that observed by fluorescence imaging. In addition, ex vivo analysis of gold quantity at the tumor site closely correlated with the fluorescence intensity recorded 72 h post-transplantation, further establishing that the NK92 cells were indeed those being imaged in the tumor over time.

Given the wide applicability of our GNP-based cell-tracking concept, the ability to track NK cells can be further applied to state-of-the-art chimeric antigen receptor (CAR)-modified NK cell products. As opposed to identifying the target cells independent of the T cell receptor and the major histocompatibility complex, CAR-modified cells enable identification of antigens expressed upon the target cell surface, including proteins, carbohydrates, and glycolipids. Although CAR-modified T cell therapy has led to a dramatic clinical response in hematological malignancies, it entails many logistic limitations as a personalized treatment and cannot be used as an allogeneic product, due to the high risk of graft-versus-host disease. The use of allogeneic NK cells can circumvent these limitations and therefore NK cells (whether primary or NK92) may be used as an “off-the-shelf” cellular source for immunotherapy. The next-generation engineered NK cells show great promise in preclinical research, and several CAR-NK-based clinical trials are underway.

Recent studies have also attempted to determine the NK cell dosage to be used to treat cancer patients with different tumor types and disease severity. Thus, we trust that the present tracking approach could facilitate the identification of optimal and personalized treatment conditions.

In summary, we present a novel method for longitudinal and quantitative in vivo NK cell tracking, based on the superior visualization ability of CT, combined with the state-of-the-art nanotechnology. NK cell in vivo visualization can solve previously unanswered questions regarding the fate of cells and allow for the real-time evaluation of their functions in clinical applications. We trust that a precise and reliable ability to track native and genetically engineered NK cells will greatly facilitate the safe implementation of this kind of promising immunotherapy.

### MATERIALS AND METHODS

**GNP Synthesis, Conjugation, and Characterization.** GNPs were prepared using sodium citrate according to the known methodology described by Enunst and Turkevich. First, 0.414 mL of 1.4 M HAuCl\(_4\) solution in 200 mL of water was added to a 250 mL single-neck round-bottom flask and stirred in an oil bath on a hot plate until boiling. Then, 4.04 mL of 10% sodium citrate solution (0.39 M sodium citrate tribasic dihydrate 98%, Sigma CAS 6132-04-3) was quickly added. The solution was stirred for 5 min and then the flask was removed from the hot oil and placed aside until it cooled.

To prevent aggregation and stabilize the particles in physiological solutions, O-(2-carboxyethyl)-O-(2-mercaptoethyl) heptaethylene glycol (PEG7) (95%, Sigma-Aldrich, Israel Ltd.) was adsorbed onto the GNPs. This layer also provided the chemical groups required for further conjugation (−COOH). First, the solution was centrifuged to dispose of excess citrate. PEG7 solution was then added to the GNP solution, stirred overnight, and put in a centrifuge in order to dispose of excess PEG. To increase the cell-uptake rate, stabilized GNPs were further coated with glucose. Excess N-ethyl-N-(3-(dimethylamino)-propyl) carbodiimide (EDC) and...
N-hydroxysuccinimide (NHS) (Thermo Fisher Scientific, Inc., Rockford, IL) were added to the solution, followed by the addition of glucose-2 (2GF) (d(-)-glucosamine hydrochloride, Sigma-Aldrich Israel Ltd.). NHS and EDC form an active ester intermediate with the —COOH functional groups, which can then undergo an amidation reaction with the glucose -NH₂ group. Glucosamine molecule C-2 (2GF-GNP): d(-)-glucosamine hydrochloride (3 mg; Sigma-Aldrich) was added to the activated linker-coated GNPs. TEM (JEM-1400, JEOL) was used to measure the size and shape of the GNPs, which were further characterized using UV–vis spectroscopy (UV-1650 PC; Shimadzu Corporation, Kyoto, Japan) following each level of coating.

**Cell Lines.** NK92 cells were a kind gift from Prof. Angel Porgador from the Faculty of Health Sciences at Ben-Gurion University. The melanoma cell line 888 was cultured in DMEM and the K562 cell line was cultured in RPMI (Biological Industries, Beth Haemek, Israel), supplemented with 10% heat-inactivated fetal bovine serum (FBS; Biological Industries, Beth Haemek, Israel), and maintained in a 5% CO₂ incubator at 37 °C. NK92 cells were cultured in α-MEM medium (Biological Industries, Beth Haemek, Israel) supplemented with 15% FBS, 15% horse serum, 10% myo-inositol (Biological Industries, Beth Haemek, Israel), 10% MEM sodium pyruvate (Biological Industries, Beth Haemek, Israel), and 200 IU/mL IL-2 and maintained at 37 °C in a 5% CO₂ incubator.

**Cytokine Release Assay.** NK92 cell cultures were tested for reactivity by the cytokine release assay performed using a commercially available ELISA kit for detecting IFN-γ (R&D Systems, Minneapolis, MN). For this assay, 1 × 10⁵ responder cells (NK92) and 1 × 10⁶ stimulator cells (tumor cells) were incubated in a 0.2 mL culture volume in individual wells of 96-well plates. Stimulator cells and responder cells were cocultured for 18 h. Cytokine secretion was measured in culture supernatants diluted to be in the linear range of the assay.

**Cell-Mediated Cytotoxicity Assay.** Target cells were labeled with 2 mM CFSE (ebioscience, San Diego, CA) for 6 min and then co-cultured with labeled lymphocytes at 37 °C for 4 h, at an E/T ratio of 3:1. After co-culture, 1 mM propidium iodide (PI; Sigma-Aldrich Israel Ltd.) was added for assigning the ratio of cell death. Samples were analyzed by flow cytometry.

**Cell Cycle Analysis.** NK92 cells were loaded with GNP by incubation in saline for 1 h with gold at a concentration of 0.76 mg gold/mL. The cells were then washed from gold residues and transferred to medium for another 48 h. Next, the cells were harvested and washed twice in cold PBS and fixed for 24 h at 4 °C, using 4 mL of cold 70% ethanol. For PI staining and flow cytometry analysis, the fixed cells were washed and centrifuged at 500g for 5 min. Cell pellets were then resuspended in 400 mL of PBS supplemented with 8 mL of RNase (1 mg/mL) and 4 mL of PI (2 mg/mL). Samples were incubated for 10 min in the dark, before being analyzed by flow cytometry.

**Flow Cytometry Analysis and Antibodies.** Immunofluorescence of anti-human CD56 (BioLegend, San Diego, CA) and fluorescein-labeled antibodies, analyzed as the relative log fluorescence of live cells, was measured using a CyAn-ADP flow cytometer (Beckman Coulter, Brea). Approximately, 1 × 10⁶ cells were analyzed. Cells were stained in the FACS buffer made of PBS, 0.5% BSA, and 0.02% sodium azide.

**Animal Model and Injections.** 6 week old NOD SCID gamma (NSG) male mice (Harlan, Jerusalem, Israel) were subcutaneously injected with 2 × 10⁶ 888 or K562 cells (12 mice in each group). After tumor establishment (7–10 days), mice were divided into three groups, and 10 × 10⁶ GNP-loaded NK92 lymphocytes were intravenously injected into the mice. Tumor growth measurements were conducted in a blinded fashion using a caliper and calculated using the following formula: [D × d₂] × π/6, where D is the largest tumor diameter and d its perpendicular one. All procedures were performed according to the guidelines of the University Committee for Animal Welfare.

**Live Fluorescence Imaging.** Fluorescence was examined by the Maestro II in vivo imaging system, a 2D planar fluorescence imaging of small animals (Cambridge Research & Instrumentation, Inc., Woburn, MA). A deep red excitation/emission filter set was used for our experiments (λₑ: 641–681 nm, λₑ: >700 nm). The liquid crystal tunable filter (LCTF) was programed to acquire image cubes with an increment of 10 nm per image. Fluorescence intensity measurements were calculated as the average intensity over the tumor surface area, using ImageJ software.

**Flame Gold Analysis.** For analyzing the amount of GNPs within the cells, the cells were incubated with GNPs (30 mg/mL) for 2 h. The cells were melted with aqua regia, a mixture of nitric acid and hydrochloric acid in a volume ratio of 1:3. The uptake was analyzed using flame atomic absorption spectroscopy (FAAS, SpectrAA140), Agilent Technologies.

For the biodistribution assay, tumor-bearing mice treated with GNP-loaded NK cells were sacrificed at 24, 48, and 72 h after injection, and their organs were excised and analyzed for the gold content using FAAS. The organs (tumor, lungs, liver, spleen, kidneys, and blood) were melted with 1 mL of aqua regia and then evaporated and diluted to a total volume of 10 mL. A Au lamp was used in order to determine the gold concentration in the samples. The gold concentration in each sample was determined according to its absorbance value with correlation to a calibration curve. Each sample was analyzed in triplicate and averages and standard deviations were taken.

**In Vivo Micro-CT Scans.** In vivo scans were performed using a micro-CT scanner (SkyScan high-resolution model 1176) with a nominal resolution of 35 μm, a 0.2 mm aluminum filter, and a tube voltage of 45 kV. Reconstruction was done with a modified Feldkamp39 algorithm using SkyScan NRecon software accelerated by GPU. Ring artifact reduction, Gaussian smoothing (3%), and beam-hardening correction (20%) were applied. 2D images and quantitative measurements were obtained using CT-Analyze (“CT-An”) software. Volume-rendered 3D images were generated using an RGBA transfer function in both SkyScan CT-Volume (“CTVol”) and CT-Voxel (“CTVox”) software.

**Statistical Analysis.** Comparison of the tumor growth rate and of data between two groups were performed using paired two-tailed Student’s t-test. The p-values below 0.05 were considered significant.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c02143.

CT images of GNP-labeled cell accumulation in the tumor over time (PDF)
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Notes
The authors declare no competing financial interest.

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