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Pilot study of a novel nanobody 68Ga-NODGAG-SNA006 for instant PET imaging of CD8+ T cells

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Research Article

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Abstract

Purpose Positron emission tomography (PET) with specific diagnostic probes for quantifying $CD8^+$ T cells has emerged as a powerful technique for monitoring immune response. However, currently, most $CD8^+$ T cell radiotracers are based on antibodies or antibody fragments, which are slowly cleared from circulation. Herein, we aimed to develop and assess ^{68}Ga-NODAGA-SNA006 for instant PET (iPET) imaging of $CD8^+$ T cells.

Methods A novel nanobody without a hexahistidine (His_6) tag, SNA006-GSC, was designed and site-specific conjugated with NODAGA-maleimide and radiolabeled with ^{68}Ga. The PET imaging profiles of ^{68}Ga-NODAGA-SNA006 were evaluated in BALB/c MC38-$CD8^+$/$CD8^-$ tumor models and cynomolgus monkeys. Three lung cancer volunteers underwent whole-body PET/CT imaging after ^{68}Ga-NODAGA-SNA006 administration. Biodistribution, pharmacokinetics and dosimetry of patients were also investigated. In addition, combined with immunohistochemistry (IHC), the quantitative performance of the tracer to monitor CD8 expression was evaluated in BALB/c MC38-$CD8^+$/$CD8^-$ and human subjects.

Results ^{68}Ga-NODAGA-SNA006 was prepared with RCP>98% and SA>100 GBq/μmol. ^{68}Ga-NODAGA-SNA006 exhibited specific tumor uptake in MC38-$CD8^+$ tumors of xenografts, CD8-rich tissues (such as the spleen) of monkeys and $CD8^+$ tumor lesions of patients within 1 h. Fast washout from circulation appeared in three volunteers ($t_{1/2}$<20 min). A preliminary quantitative linear relationship (R^2=0.9668, p<0.0001 for xenografts and R^2= 0.7924, p=0.0013 for lung patients) appeared between ^{68}Ga-NODAGA-SNA006 uptake and CD8 expression. ^{68}Ga-NODAGA-SNA006 was well tolerated in all patients.

Conclusion ^{68}Ga-NODAGA-SNA006 PET imaging can instantly quantify CD8 expression with an ideal safety profile and is expected to be important for dynamically tracking $CD8^+$ T cells and monitoring immune responses for individualized cancer immunotherapy.

Trial Registration NCT05126927 (19 November 2021, retrospectively registered).

Introduction

Immunotherapy has brought revolutionary breakthroughs to many cancers. However, only a subset of melanoma patients benefit from immunotherapy, which has even lower efficiency for most solid cancers and a variable effect [1, 2]. Therefore, there is an urgent need for a priori screening for precise responders and timely adjustments to treatment strategies during the immunotherapy process to maximize the benefits to oncology patients [3]. The quantity and quality of immune cells in the tumor microenvironment have a significant prognostic impact on a wide range of cancers [4], which is the so-called “Central Dogma for Immunotherapy” [5].

Cytotoxic $CD8^+$ T cells are the most powerful effectors in the anticancer immune response and are considered potential predictive biomarkers for successful cancer immunotherapies [6–9]. There is a

clinical need for the accurate determination of the $CD8^+$ T cell status of tumors because of its prognostic value and the need for the identification of patients who would benefit from immunotherapies [10, 12]. Currently, the two main methods for the detection of $CD8^+$ T cells are lymphocytes from whole blood and biopsies from heterogeneous tumors. However, evaluation of the dynamic and spatial profiles of $CD8^+$ T cells is still challenging [10]. Serial whole-body positron emission tomography combined with computed tomography (PET/CT) imaging using specific tracers is an ideal tool to dynamically monitor both systemic and intratumoral alterations in $CD8^+$ T cell numbers and perform localization during immunotherapies [9]. This noninvasive approach can reveal critical information regarding tumor response to enable informed clinical decisions.

To date, numerous radiolabeled antibodies and antibody fragment-labeled radionuclides used for $CD8^+$ T cell targeted PET tracers have been developed in both preclinical and clinical studies [8, 11–15], such as ^{89}Zr-IAB22M2C (anti-CD8 minibody) [16]. Minibody (80 kDa) with terminal half-lives in mice of approximately 5–10 h was a rapid targeting and faster clearance than intact antibodies (~ 150 kDa). Nevertheless, it also mandates the use of a relatively long-lived radionuclide, i.e., ^{89}Zr [16–18] or ^{64}Cu [11, 12, 19], which was scanned by PET at least 24 h after probe injection and brought a high radiation dose to the patient, [20, 21]. Smaller antigen-binding fragments derived from conventional antibodies, such as Fabs (~ 55 kDa) and scFvs (~ 25 kDa), exhibit shorter blood half-lives but tend to lack sufficient stability and solubility, resulting in increased nonspecific uptake by nontarget organs [22, 23]. Therefore, many challenges exist in instantly monitoring the tumor infiltration degree of $CD8^+$ T cells with these tracers [24].

Accordingly, efforts to develop small-molecule alternative antibody formats have been accelerated. Single-domain antibody fragments (~ 15 kDa), also referred to as nanobodies, are one of the most attractive alternatives due to their superior imaging characteristics, such as fast targeting, high signal-to-background ratios, and ideal stability [9, 25]. More importantly, only a short half-life radionuclide, i.e., ^{18}F or ^{68}Ga, is required for radiolabeling to match its rapid blood clearance. Accordingly, these radiolabeled nanobody agents can allow for instant imaging and dynamic monitoring of the kinetics of tumor-infiltrating $CD8^+$ T cells, which is more convenient for patients and can be used as an ideal companion diagnostic for clinical transformation.

Previously we constructed a ^{68}Ga-labeled nanobody tracer, ^{68}Ga-NOTA-SNA006a, with high targeting specificity to human CD8 protein (KD = 0.64 nM) [8]. In this study, we optimized the structure of the nanobody by removing the His_6 tag to prepare SNA006-GSC and developed the site-specific tracer ^{68}Ga-NODAGA-SNA006. We aimed to evaluate the instant PET imaging profiles of ^{68}Ga-NODAGA-SNA006 in rodent tumor models, nonhuman primates, and first lung cancer volunteers. More importantly, combined with immunohistochemistry (IHC), we evaluated the quantitative sensitivity of the tracer to monitor CD8 expression in BALB/c MC38-$CD8^+$/$CD8^-$ tumor models as well as human subjects.

Materials And Methods

Detailed descriptions of all reagents, experiments, materials, and techniques can be found in the Supplementary Material (SM), together with additional supporting tables and figures. All animal experiments were performed in accordance with the guidelines of Soochow University Institutional Animal Care.

Conjugation andRadiolabeling

The novel SNA006-GSC nanobody was provided by SmartNuclide Biopharma (Suzhou, China). Anti-CD8α nanobody DNA fragments were re-cloned in a suitable mammalian expression vector pcDNA4 (Invitrogen, Cat V86220). Other details of SNA006-GSC nanobody preparation methods were refer to our previous reports [26]. Cysteine-containing sequence (GSC) at the C-terminus of SNA006 was used to conjugated with NODAGA-maleimide site-specifically.

SNA006-GSC nanobody was conjugated to NODAGA-maleimide as previously described with some modifications [27]. Briefly, tris (2-carboxyethyl) phosphine hydrochloride (TCEP) solution was mixed with SNA006-GSC to open the disulfide bond formed by cysteine at the end of the protein. Excess TCEP and free cysteine were removed by a 3 kDa ultrafiltration tube. NODAGA-maleimide (1.2 eq) solution was mixed with SNA006-GSC (1 eq) and reacted at 37°C for 24 h. After the reaction, the superfluous NODAGA-maleimide was removed by an ultrafiltration tube. Characterization of NODAGA-SNA006 was performed by size exclusion-high-performance liquid chromatography (SEC-HPLC) and liquid chromatograph-mass spectrometer (LC-MS).

For ^{68}Ga radiolabeling, $^{68}GaCl_3$ solution (1 mL) was eluted from a ^{68}Ge/^{68}Ga generator with 0.05 M HCl (Merck), and then an aqueous sodium acetate solution (0.25 M, 400 μL) and NODAGA-SNA006 (100 μg) precursors were added to the system (pH=4). The reaction was incubated at 37°C for 15 min and purified with a PD-10 column (Cytiva) to obtain ^{68}Ga-NODAGA-SNA006 in a saline solution.

Radio-HPLC with water and acetonitrile (both water and acetonitrile contained 0.1% trifluoroacetic acid) as the mobile phase (1 mL/min) was used to analyze the radiochemical purity (RCP) of ^{68}Ga-NODAGA-SNA006.

In vitro biological performance studies of SNA006-GSC

The affinity of SNA006-GSC and NODAGA-SNA006 binding to human CD8 protein and PBMC as well as the in vitro safety assessment of SNA006-GSC to PBMC were performed with surface plasmon resonance (SPR), ELISA Reader (MD-SpectraMax P190) or Flow cytometer (Beckman Coulter CytoFLEX S). Detailed methods are offered in SM.

In vitro and in vivo stability of ^{68}Ga-NODAGA-SNA006

The in vitro stability of ^{68}Ga-NODAGA-SNA006 in saline solution and in human serum (37°C) as well as the in vivo stability in ICR mice were also determined with radio-HPLC. Detailed methods are offered in SM.

Micro-PET imaging in BALB/c MC38-CD8$^+$/CD8$^-$ xenografts

Six BALB/c mice with subcutaneous MC38-CD8$^-$ (left) and MC38-CD8$^+$ (right) xenografts were injected via the tail vein with 2.28±0.07 MBq of ^{68}Ga-NODAGA-SNA006, and immediately, dynamic PET imaging was performed within the first 1 hour. Then, 10-minute PET scans were acquired at 2 and 4 h postinjection (p.i.). All micro-PET scans were performed on an Inveon micro-PET scanner (Siemens). The ordered subset expectation maximization 3D (OSEM3D) algorithm was used to reconstruct the PET data. The percentage injected dose per gram of tissue (%ID/g) in regions of interest (ROIs) was analyzed by the Inveon Research Workplace ASIPro (Siemens Medical Solution) workstation.

Biodistribution and pharmacokinetics in BALB/c MC38-CD8$^+$ /CD8$^-$ xenografts

Sixteen BALB/c mice with subcutaneous MC38-CD8$^-$ and MC38-CD8$^+$ xenografts were used for the biodistribution and pharmacokinetics study. Mice were intravenously injected with 1.24±0.45 MBq of ^{68}Ga-NODAGA-SNA006 and sacrificed at 0.5, 1, 2 and 4 h p.i. (n = 4 per group). Among them, for the 4 h group models, 10-20 μL of blood were collected from the tail at 5, 10, 15, 30, 60, 90 and 120 min for pharmacokinetics investigation. MC38-CD8$^-$ and MC38-CD8$^+$ tumors and other organs as well as blood samples were collected, weighed immediately, and detected with a gamma counter (2480 WIZARD2, Perkin Elmer). The tissue uptake (%ID/g) can be calculated. A noncompartmental analysis was performed for the blood uptake using linear trapezoidal fitting with Phoenix WinNonLin (version: 6.4).

Autoradiography and immunohistochemistry

To further verify the tumor radioactive uptake and CD8 expression in the corresponding tumor microenvironment in tumor models, ex vivo autoradiography and CD8 immunohistochemistry (IHC) were also performed in the above distribution animal models at 2 h after administration of ^{68}Ga-NODAGA-SNA006. Detailed methods are offered in SM.

Quantitative performance of ^{68}Ga-NODAGA-SNA006 in assessing CD8 expression in tumors

Eighteen tumor-bearing models that simulated different CD8 expression levels with various MC38-CD8$^+$ cell rations (0, 20%, 40%, 60%, 80%, 100%) were used to assess the quantitative performance of ^{68}Ga-NODAGA-SNA006. In total, each model was administered intravenously with 2.02±0.75 MBq of ^{68}Ga-NODAGA-SNA006 and 10-minute PET imaging was performed at 1 h p.i.

After PET imaging, tumors were harvested and fixed in 4% buffered formalin solution for 7 days, and used for hematoxylin/eosin (H&E), immunofluorescence and IHC staining of CD8 (Abcam). The

quantitative performance of ^{68}Ga-NODAGA-SNA006 was analyzed by studying the correlation of tumor uptake from PET imaging and IHC. Detailed methods are offered in SM.

PET/CT imaging and safety evaluation in monkeys

Two male cynomolgus monkeys were used for ^{68}Ga-NODAGA-SNA006 PET/CT imaging with a GE Discovery 16 PET/CT scanner (GE Healthcare). Approximately 45-55 MBq ^{68}Ga-NODAGA-SNA006 was injected via the small saphenous vein of the lower extremities. One cynomolgus monkey was administered a mass of 25 μg/kg SNA006-GSC, and the other monkey was administered a higher dose of 150 μg/kg SNA006-GSC. Whole-body PET/CT scans were acquired immediately after administration and delayed imaging at 0.25, 0.5, 1, 1.5, 2 and 4 h p.i.

Approximately 1.5 mL of blood samples were collected from the above two cynomolgus monkeys before and after administration at 24 h, 7 d and 14 d p.i.. The blood biochemical indicators, routine indicators, coagulation indicators and anti-drug antibodies were analyzed.

Patients study of ^{68}Ga-NODAGA-SNA006

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the Helsinki Declaration. This FIH study was approved by the Research Ethics Board of the First Affiliated Hospital of Soochow University and all patients signed a written informed consent form before study participation (ClinicalTrials.gov identifier NCT05126927). Three lung cancer patients (1 woman and 2 men; mean age±SD, 68.3±4.0 y) were enrolled in this study (Table S1), among them, one patient finished three courses of immunotherapy (sintilimab) combined with chemotherapy (paclitaxel and carboplatin).

Three lung cancer volunteers underwent ^{68}Ga-NODAGA-SNA006 in a GE Discovery 16 PET/CT scanner (GE Healthcare). Patients received an intravenous injection of ^{68}Ga-NODAGA-SNA006 (163.8 ± 9.8 MBq, 100 μg for the first two patients and 800 μg for the third one) into their arms. Each patient underwent 3 whole-body PET/CT scans from the vertex of the skull to femur at 15-30 min, 60-90 min, and 120 min p.i. A single CT scan at 15-30 min p.i., was obtained with 120 mA tube current (140 kVp; estimated radiation dose 7.6 mGy), while all other low-dose CT scans were performed with a 10 mA current (120 kVp; estimated radiation dose 0.6 mGy).

Detailed methods of PET/CT imaging analysis, pharmacokinetics of patients as well as immunohistochemistry of lesions after surgery are offered in SI.

Safety and Normal Organ (Tissue) Dosimetry in human subjects

Adverse events (AEs) were described according to the National Cancer Institute Common Terminology Criteria for Adverse Events (CTCAE) grading system (version 5.0). Heart rate, blood pressure and pulse oximetry were recorded within 1 h before and at 2 and 24 h after ^{68}Ga-NODAGA-SNA006 injection. Fresh

specimens of tumor tissue or adjacent normal areas from the tumor edge of three cancer patients were immediately taken after the surgery used for IHC.

According to a previous report [28], the whole-body normal organ effective dose was determined with OLINDA/EXM software (version 2.1) using adult female models for women and adult male models for men.

Statistical Analysis

GraphPad Prism (version 8) was used for statistical analyses of the data. Quantitative data are expressed as the mean ± standard deviation (SD), with all error bars denoting the SD. The means were compared using Student's t test, and P values less than 0.05 were considered statistically significant.

Results

Conjugation and Radiolabeling

The terminal cysteine of the single-domain antibody SNA006-GSC was site-specific conjugated with NODAGA-maleimide as SEC-HPLC and LC-MS results show in Fig. S1. The characterization results of NODAGA-SNA006 indicated that each chelating agent molecule was coupled with a single-domain antibody molecule with a fixed conjugation ration=1.

^{68}Ga-NODAGA-SNA006 was prepared with high radiochemical yield (> 95%), RCP > 98% and specific activity (SA>100 GBq/μmol).

In vitro biological performance studies

Conjugation with NODAGA-maleimide did not affect the in vitro performance of SNA006-GSC, both SNA006-GSC and NODAGA-SNA006 exhibited a strong affinity for human CD8 protein with a relatively low equilibrium dissociation constant (KD), approximately 1.01×10^{-9} M for SNA006-GSC (Fig. S2a) and 1.90×10^{-9} M for NODAGA-SNA006 (Fig. S2b). In addition, the typical S-type binding curve between SNA006-GSC and PBMCs was observed in the binding experiment, and half of the effective concentration (EC_{50}) fitted from this curve was approximately 0.522 nM (Fig. S2c). Furthermore, the existence of SNA006-GSC did not affect the viability, proliferation or excitation of PBMCs, which suggested that it obtained an ideal in vitro safety performance (Figs. S2d and S2e).

In vitro and in vivo stability of ^{68}Ga-NODAGA-SNA006

^{68}Ga-NONAGA- SNA006 was observed to have excellent in vitro stability in normal saline solution and serum with RCPs > 95% over 4 h (Figs. S3a and S3b).

As shown in Fig. S3c, ^{68}Ga-NODAGA-SNA006 in mouse blood was also observed to have an ideal stability with RCP>90% over 30 minutes, while the radioactivity in the blood was too low to detect at the

subsequent time point due to physical decay and biological metabolism. Most of the radioactive substances excreted from the urine exist in the prototype form of the radio probe, as more than 78% of ^{68}Ga-NODAGA-SNA006 was still present in the urine 4 h p.i. (Fig. S3d).

Micro-PET imaging and biological behavior in BALB/c MC38-CD8$^+$ /CD8$^-$ models

From Micro-PET imaging in Fig. 2a, ^{68}Ga-NODAGA-SNA006 accumulated rapidly in MC38-CD8$^+$ tumors, while MC38-CD8$^-$ tumors were barely visible. This was consistent with the biodistribution results. The uptake in MC38-CD8$^+$ tumors was 9.06±0.83% ID/g at 30 min after injection, while for the MC38-CD8$^-$ tumors, the uptake was 0.62±0.03%ID/g. The ratio of MC38-CD8$^+$/MC38-CD8$^-$ tumor uptake continued to increase from 9.31±0.27 at 1 h to 42.19±21.54 at 4 h on Micro-PET imaging (Fig. 2b).

In addition to biodistribution, an autoradiography experiment was performed simultaneously with CD8 IHC (Fig. 2c) and proved the high ratio of MC38 CD8$^+$/CD8$^-$ tumor uptake. For example, at 2 h p.i., the ratio was 21.85 according to autoradiography (Fig. 2d) and similar to Micro-PET imaging result with 22.54±4.55 (Fig. 2b).

The tracer is mainly excreted through the kidneys. Among all organs, the kidney uptake was much higher than that in other tissues in the BALB/c tumor-bearing model. The biodistribution results showed that kidney uptake was highest at 1 h after injection, with 79.58±20.74% ID/g, followed by MC38-CD8$^+$ tumors (8.92±2.94% ID/g), while there were no abnormal radioactive concentrations in other normal tissues, such as the liver, spleen, lung and brain.

Fig S4 shows the curve of blood uptake with time, which suggested that ^{68}Ga-NODAGA-SNA006 was rapidly cleared from the circulatory system with a half-life of 25.8±5.4 min.

Quantitative sensitivity of ^{68}Ga-NODAGA-SNA006 for detecting MC38-CD8$^+$ cells in vivo

Images of ^{68}Ga-NODAGA-SNA006 in tumor models inoculated with different proportions of MC38-CD8$^+$ cells are presented in Fig. 3a. Although there were individual differences in tumor uptake with the same MC38-CD8$^+$/CD8$^-$ cell ratio, the overall trend was that tumors with high proportions of MC38-CD8$^+$ cells had high ^{68}Ga-NODAGA-SNA006 uptake. Tumors were inoculated with 100% MC38-CD8$^-$ cells, and the ^{68}Ga -NODAGA-SNA006 imaging uptake value was 0.40% ID/g, with negative CD8 IHC staining. For tumors with an uptake value of 4.16% ID/g, the density of CD8 expression was 33.80±6.59%, and for tumors with an uptake value of 8.51%ID/g, the CD8 density was 64.43±4.81%.

Despite the tumor heterogeneity, we found that the higher tumor uptake of ^{68}Ga-NODAGA-SNA006 on imaging means higher CD8 expression. Further analysis showed a quantitative linear relationship (R^2=0.9668, p<0.0001) between the tumor uptake of ^{68}Ga-NODAGA-SNA006 and the mean density of CD8 expression within the tumor (Fig. 3c). The tumor tissue grew normally without necrotic architecture according to H&E staining (Fig. 3b).

PET/CT imaging in monkeys

Maximum intensity projection (MIP) PET/CT images at multiple time points after intravenous injection are shown in Fig. 4a. Similar to rodents, the tracer was mainly excreted through the kidneys and bladder in cynomolgus monkeys. Low background activity was seen in CD8-poor tissues such as brain, lung, and muscle in both dose groups (25 and 150 μg/kg). ^{68}Ga-NODAGA-SNA006 accumulated in CD8-rich tissues (e.g. spleen, bone marrow, and lymph nodes) with high, rapid and continuous uptake during the 4 h monitoring period for the lower dose of 25 μg/kg (Figs. 4d and e).

The spleen had the highest standardized uptake value (SUV) at all acquisition times, with values of 37.05 and 37.65, reaching 191-to-1 and 438-to-1 ratios compared with muscle at 1 h and 2 h p.i. Furthermore, accumulation in the CD8-rich spleen and bone marrow was obviously decreased for the higher dose of 150 μg/kg (Figs. 4b and c). Spleen uptake was reduced by more than 90% and the spleen-to-muscle ratio decreased to 13:1 and 14:1 at 1 h and 2 h p.i., respectively.

It was safe and well tolerated in monkeys in both the 25 μg/kg and 150 μg/kg groups.

First-in-human PET/CT imaging of ^{68}Ga-NODAGA-SNA006

No adverse events or the results of clinical laboratory tests were found for any of the volunteers during the PET imaging procedure or within 1 wk after injection of ^{68}Ga-NODAGA-SNA006 (163.8± 9.8 MBq).

The spleen showed the highest radioactivity accumulation, followed by the bladder. Consistent with preclinical results, ^{68}Ga-NODAGA-SNA006 was excreted primarily through the kidney. The liver was the third highest uptake (SUV_{max} of 12.03 and 16.11) in the low-dose group (100 μg), and significantly decreased (SUV_{max} of 6.36) with increasing dose (800 μg) at 60 min (Fig. 5). ^{68}Ga-NODAGA-SNA006 was also concentrated in the bone marrow, whereas the heart, normal lung tissue, brain, and muscle were relatively low (Table S2).

In the first lung adenocarcinoma patient with a lesion of 20×21 mm, the tumor uptake of ^{68}Ga-SNA006-NODAGA was evident, with SUV_{max} of 1.09 (15 min), 1.19 (60 min) and 1.78 (120 min), while the normal lung uptake was 0.50 (15 min), 0.63 (60 min) and 0.38 (120 min). Lesion uptake was nearly 2-5 times higher than that of the background (Fig. 6a). The second patient, who received immunotherapy, had higher uptake both in the lesion and the normal lung than the first patient, with an SUV_{max} of 1.39 (lesion) and 1.23 (lung) at 60 min, respectively (Fig. 6b). The third non-small cell lung cancer patient had the largest tumor lesion (55×40 mm) and obvious heterogeneity on ^{68}Ga-NODAGA-SNA006 PET imaging, with an SUV_{max} of 3.23 (60 min) for the highest concentration lesion, and 0.63 (60 min) for the lowest concentration lesion (Fig. 6c).

IHC staining confirmed the expression of CD8 in the tumor lesions and paracancerous tissue (Fig. 6d-l), suggesting that ^{68}Ga-NODAGA-SNA006 PET imaging has high sensitivity in quantifying CD8 expression.

A preliminary quantitative linear relationship (R^2= 0.7924, p= 0.0013) appeared between the tumor uptake of ^{68}Ga-NODAGA-SNA006 and the absolute $CD8^+$ T cell density (number of positive cells/mm^2) within the tumor lesions and paracancerous tissue (Fig. 6m).

As shown in Fig S4, the uptake curves of whole blood and serum over time demonstrated that ^{68}Ga-NODAGA-SNA006 was rapidly cleared from the circulatory system. The blood and serum biological half-lives of ^{68}Ga-NODAGA-SNA006 in human blood and serum fitted with WinNonlin software were 19.84±2.97 min and 18.92±3.04 min, respectively.

The absorbed dose of ^{68}Ga-NODAGA-SNA006 in the first female subject was 0.0438 mSv/MBq, and 0.0266 mSv/MBq and 0.0326 mSv/MBq for the other two male patients.

Discussion

Immunological composition and status (i.e., activated or suppressed) in a tumor microenvironment affect overall survival and dictate immunotherapeutic strategies focusing on inducing or enhancing cytotoxic $CD8^+$ T lymphocyte (CTL) infiltration within tumors. Therefore, the development of new tracers targeting $CD8^+$ T cells with iPET imaging is real-time and noninvasive imaging modality for the visualization of T-cell responses, which is of clinical importance to evaluate the immune landscape of patients prior to and during the response to immunotherapy.

Herein, we successfully developed a novel CD8-targeted iPET imaging companion diagnostic probe ^{68}Ga-NODAGA-SNA006 for human applications. SNA006-GSC was a nanobody, despite this, the binding affinity (EC_{50} = 0.522 nM) to $CD8^+$ T cells was similar to that of an IAB22M2C minibody (EC_{50} = 0.4 nM) [29]. In addition, the flow cytometry results suggested that even at high concentrations (1 μg/mL), SNA006-GSCs did not affect the viability of $CD8^+$ T cells.

SNA006-GSC was successfully conjugated to NODAGA-maleimide by site-specific modification. Compared to random modification based on lysine-linked chelators, a fixed modification position and conjugation ratio can be obtained, rather than mixtures of species with various numbers of linkers at different sites on the nanobody surface [8], which might affect the binding activity [30]. Furthermore, NODAGA-SNA006 was site-specifically labeled by ^{68}Ga with high radiochemical yield (> 95%) and SA (> 100 GBq/μmol) as well as excellent stability over 4 h. This is due to site-specific strategies offering the advantage of stronger repeatability and manufacturing a homogenous pharmaceutical product with good characterization [31]. Site-specific strategies are preferred for ^{68}Ga-NODAGA-SNA006 clinical translation.

Nanobody imaging probes are usually highly concentrated in the kidney, which is a typical feature of small hydrophilic proteins caused by efficient renal clearance and nonspecific reabsorption by proximal tubules [32]. In this study, we optimized the structure of the nanobody and removed the His_6 tag fragment. Lower kidney uptake (78.50 ± 20.74% ID/g at 1 h) appeared compared with ^{68}Ga-NOTA-

SNA006a (150% ID/g at 1 h with His6 tag) [8] in the same tumor-bearing models. We found that kidney uptake was decreased in association with the His_6 tag, which was supported by Xavier.et al [33].

An ideal diagnostic tracer should obtain the properties of efficient targeting and excellent tumor-to background ratios with high-contrast imaging. Quantitative analysis of micro PET imaging showed that ^{68}Ga-NODAGA-SNA006 has a higher $CD8^+/CD8^-$ tumor uptake ratio (21.65 ± 8.47 at 1 h) in BALB/c MC38-$CD8^+/CD8^-$ tumor models than ^{68}Ga-NOTA-SNA006a (15.14 at 1 h). In addition, the biodistribution in mouse analysis also showed fast washout of radioactivity from the blood, resulting in high tumor-to-blood (32.08 ± 10.95) at 1 h after injection, which suggested that higher image quality can be obtained with ^{68}Ga-NODAGA-SNA006. PET/CT imaging in cynomolgus also showed that ^{68}Ga-NODAGA-SNA006 was mainly retained in CD8-rich tissues, such as the spleen, bone marrow, and lymph nodes. with low uptake in CD8-poor tissues such as the brain, lung and muscle at the same drug dose. Spleen uptake reached 191-to-1 and 438-to-1 ratios compared with muscle at 1 h and 2 h p.i. Furthermore, the spleen concentration was reduced by more than 90% from 25 μg/kg to 150 μg/kg. All the above preclinical results suggested that ^{68}Ga-NODAGA-SNA006 has good CD8 targeting and specificity performance in vivo.

Next, we preliminarily evaluated the distribution and pharmacokinetics of ^{68}Ga-NODAGA-SNA006 in three cancer volunteers. ^{68}Ga-NODAGA-SNA006 was rapidly cleared from the circulatory system, and its half-life in patients was 19.84 ± 2.97 min. Even at the first ^{68}Ga-NODAGA-SNA006 imaging time, 15 minutes, the tumor site had already been significantly concentrated. Similar to rodent tumor models, $CD8^+$ tumor uptake plateaued 10 min postinjection until 240 min. For the anti-CD8 minibody probe ^{89}Zr-IAB22M2C, the median whole-body biological half-life (T1/2b) was 235 h (range 123–288 h) and the highest uptake in most lesions was seen at 24 or 48 h [16]. Combining these results, it can be concluded that ^{68}Ga-NODAGA-SNA006 cleared quickly and rapidly cleared targets the tumor site in vivo. This makes it possible to dynamically monitor CD8 expression before and during tumor immunotherapy in clinical applications.

More importantly, we systematically evaluated the in vivo quantitative performance of ^{68}Ga-NODAGA-SNA006 PET imaging for evaluating immune states in animal models and cancer volunteers. For the preclinical study, we found that the tumor uptake of ^{68}Ga-NODAGA-SNA006 and the CD8 expression of tumor sections have a linear relationship ($R^2 = 0.9668$, $p < 0.0001$) in BALB/c mice implanted with different proportions of MC38-$CD8^+/CD8^-$ cells (0, 20%, 40%, 60%, 80%, 100%). For the clinical investigation, compared with IHC staining for CD8, an ideal linearity and high correlation appeared between tumor CD8 expression and ^{68}Ga-NODAGA-SNA006 uptake ($R^2 = 0.7924$, $p = 0.0013$). However, some factors could not be neglected in the results, such as fewer samples, limited field of view in the image for IHC analysis and tumor heterogeneity. Even with these limitations, correlation between PET scan with ^{89}Zr/^{64}Cu labeled minibody and immunohistochemistry has also been reported by Griessinger et.al.[18] and Nagle et.al.[34]. Herein, we conclude that PET imaging with ^{68}Ga-NODAGA-SNA006 has a

high potential to visualize and quantify $CD8^+$ T lymphocyte infiltration in tumor lesions, which is a noninvasive approach to monitor the response to cancer immunotherapy.

However, this study had several limitations. First, the FIH dose setting was based on the guidelines of the microdosing study and only a simple high-dose study was performed. We found that the liver uptake was significantly reduced with 800 μg compared with 100 μg. More in-depth dose escalation PET imaging is needed to determine the appropriate clinical doses of ^{68}Ga-NODAGA-SNA006. Second, we tested ^{68}Ga-NODAGA-SNA006 in only 3 patients with lung cancer. This is a very small sample size, and further large-scale prospective clinical studies in patients with different types of tumors, as well as further studies correlation between the SUV of ^{68}Ga-NODAGA-SNA006 with $CD8^+$ T cell expression levels, which are necessary to evaluate the full potential of ^{68}Ga-NODAGA-SNA006 in $CD8^+$ T cell tumor-infiltration kinetics for cancer detection, patient screening, staging, and timely efficacy monitoring.

Conclusion

We conclude that ^{68}Ga-NODAGA-SNA006 as a new CD8 targeted nanobody tracer has an important criterion for clinical diagnostic tracers with fast clearance, specific tumor targeting, high-contrast imaging and ideal safety characteristics, giving it the potential to instantly monitor $CD8^+$ T cells with excellent quantitative performance. This noninvasive imaging approach could emerge as an important tool to dynamically track T cell responses and immune responses in patients and may aid in precise individualized tumor immunotherapy.

Declarations

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All animal procedures were conducted under a protocol approved by the Soochow University Institutional Animal Care.

Conflict of interest

The authors declare that they have no conflict of interest.

Author Contributions:

Yan Wang, Chao Wang and Minzhou Huang designed and performed all experiments, and wrote the manuscript. Songbin Qin, Jun Zhao, Meng Zheng, Yicong Bian, Chenrong Huang prepared ^{68}Ga-NODAGA-SNA006 and performed part of the preclinical experiments. Shibiao Sang, Hua Zhang, Linchuan Guo, Jiwei Jiang, Chun Xu, Na Dai, Yushuang Zheng and Jiajun Han completed the clinical research component. Liyan Miao, Min Yang and Tao Xu supervised the project and coordinated all researchers. All authors have reviewed and approved the manuscript.

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Figures

Figure 1

Schematic diagram of whole-body dynamic quantification of $CD8^+$ T cells using the site-specific ^{68}Ga-labeled nanobody SNA006-GSC as a diagnostic agent in clinical translational research.

Figure 2

Biological behavior of ^{68}Ga-NODAGA-SNA006 in BALB/c MC38-$CD8^+$/$CD8^-$ xenografts. **a** Dynamic ^{68}Ga-NODAGA-SNA006 PET imaging of BALB/c tumor-bearing models after the administration of 2.28±0.07 MBq of ^{68}Ga-NODAGA-SNA006, white circle: MC38-$CD8^+$ tumor (right), blue circle: MC38-$CD8^-$ tumor (left). **b** MC38-$CD8^+$/$CD8^-$ tumor uptake ratios on micro-PET imaging. **c** Ex vivo tumor autoradiography and CD8 immunohistochemistry (IHC) staining at 2 h p.i. with ^{68}Ga-NODAGA-SNA006. **d** The gray value

of MC38-CD8^{+}/CD8^{-} tumors with autoradiography. **e** Biodistribution results in BALB/c tumor-bearing models after the administration of 1.24±0.45 MBq ^{68}Ga-NODAGA-SNA006.

Figure 3

Quantitative sensitivity results of ^{68}Ga-NODAGA-SNA006 for detecting CD8 expression in mice. **a** ^{68}Ga-NODAGA-SNA006 PET imaging at 1 h of BALB/c MC38-CD8^{+}/CD8^{-} tumor models after administered with 2.02±0.75 MBq of ^{68}Ga-NODAGA-SNA006. **b** The H&E, IHC (CD8), IF staining of different CD8-positive expression tumors. **c** The linear relationship between tumor uptake of ^{68}Ga-NODAGA-SNA006 and the expression of CD8 in tumor tissues (R^2=0.9668, p<0.0001).

Figure 4

Dynamic PET/CT imaging of cynomolgus monkeys. **a** Maximum intensity projection (MIP) PET/CT images of monkeys after administration of 45-55 MBq ^{68}Ga-NODAGA-SNA006 with 25 μg/kg and 150 μg/kg SNA006-GSC. **b** and **c** Uptake of ^{68}Ga-NODAGA-SNA006 in the spleen and marrow analyzed according to quantification analysis of the PET/CT images. **d** and **e** Tissue uptake of ^{68}Ga-NODAGA-SNA006 in monkeys administered with 25 μg/kg and 150 μg/kg SNA006-GSC.

Figure 5

Multiple-time-point coronal tomograpphy of the tumor site in three lung cancer volunteers after the injection of ^{68}Ga-NODAGA-SNA006. Tumors are indicated by white arrows. Among these volunteers, the second patient received immunotherapy, while the other two patients did not receive any treatment.

Figure 6

^{68}Ga-NODAGA-SNA006 quantities human CD8^{+} T cells in patients. **a-c** Transverse PET/CT imaging at 1 h after ^{68}Ga-NODAGA-SNA006 administration. **d-i** IHC staining (scale bars: 5 mm(d-f) and 250 μm(g-i)) and **j-l** H&E staining (scale bars: 250 μm) of 3 patients' tumor lesions. **m** The preliminary quantitative linear relationship (R^2=0.7924, p=0.0013) between tumor uptake of ^{68}Ga-NODAGA-SNA006 on PET imaging and the absolute CD8^{+} T cell density (number of positive cells/mm^2) on CD8 IHC staining.

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