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⁸⁹Zr-labeled nivolumab for imaging of T-cell infiltration in a humanized murine model of lung cancer

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Abstract

Purpose—Nivolumab is a human monoclonal antibody that is specific for programmed cell death-1 (PD-1), a negative regulator of T-cell activation and response. Acting as an immune checkpoint inhibitor, nivolumab binds to PD-1 expressed on the surface of many immune cells and prevents ligation by its natural ligands. Nivolumab is only effective in a subset of patients and there is limited data supporting its use for diagnostic, monitoring, or stratification purposes.

Methods—⁸⁹Zr-Df-nivolumab was synthesized to map the biodistribution of PD-1-expressing tumor infiltrating T-cells *in vivo* using a humanized murine model of lung cancer. The tracer was developed by radiolabeling the antibody with the positron emitter zirconium-89 (⁸⁹Zr). Imaging studies were validated by *ex vivo* biodistribution studies, and PD-1 expression was validated by immunohistochemistry studies. Data obtained from PET imaging were used to determine human dosimetry estimations.

Results—The tracer showed high binding to stimulated PD-1 expressing T-cells both *in vitro* and *in vivo*. PET imaging of ⁸⁹Zr-Df-nivolumab allowed for clear delineation of subcutaneous tumors through targeting of localized activated T-cells expressing PD-1 in the tumors and salivary glands of humanized A549 tumor-bearing mice. In addition to the tumors, salivary and lacrimal gland infiltration of T-cells was noticeably visible and confirmed via histological analysis.

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Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Conclusions—These data support our claim that PD-1-targeted agents may allow for tumor imaging *in vivo*, which may assist in the design and development of new immunotherapies. In the future, noninvasive imaging of immunotherapy biomarkers may assist in disease diagnostics, disease monitoring, and patient stratification.

Keywords

Nivolumab; programmed cell death 1 (PD-1); positron emission tomography (PET); cancer; zirconium-89 (^{89}Zr); immunotherapy; immune checkpoint inhibitor; immuno PET

Introduction

Programmed cell death 1 (PD-1) is an inhibitory costimulatory molecule expressed on the surface of some activated T-cells, B-cells, dendritic cells, and macrophages [1]. It is a member of the B7-CD28 family of proteins and performs fundamental roles in host immunity, tolerance, and tumor evasion. Activation of PD-1 occurs through binding of either of its two natural ligands, programmed death ligand 1 or 2 (PD-L1 or PD-L2), which are expressed on the surface of many antigen presenting cells and tumor cells from various malignancies arising from the breasts, ovaries, kidneys, bladder, and lungs [2]. The interactions between PD-1 and its natural ligands negatively regulates T-cell activation and effector function through downregulation of certain antiapoptotic pathways and proinflammatory cytokines [3]; thus, diminishing the immune system's natural defense mechanism and allowing for continued tumor growth and disease progression [4]. Immunotherapies targeting this interaction leads to increased anti-tumor activities, including restoration of T-cell function and expansion, enhancement of natural killer and cytotoxic T-lymphocytes response, and increased cytokine secretion [5-8]. The use of monoclonal antibodies as immune checkpoint inhibitors has become a common strategy for many treatment-experienced patients and is currently being explored for treatment-naïve patients in some cancers [9]. Despite leading to significant improvements in patient survival and overall outcomes, clinical data reveal that only a small subset of patients respond to anti-PD-1/PD-L1 blockade therapies when used as single agents [10-12].

Nivolumab (Opdivo®) and pembrolizumab (Keytruda®) are the only anti-PD-1 immunotherapies approved by the Federal Drug Administration in the United States as of 2017 [13]. Both of these therapies have shown durable remission and prolonged survival in some patients. For example, advanced non-small cell lung cancer patients treated with nivolumab showed a 16% overall 5-year survival rate, nearly 4-times higher than that of patients receiving traditional chemotherapeutics [14]. Despite showing efficacy in some patients, severe and sometimes fatal adverse reactions have been associated with nivolumab treatment, including autoimmune-mediated organ toxicities, upper respiratory infections, edema, pruritus, and skin infections [15]. Hence, some organizations have recommended the screening of patients before initiation of PD-1/PD-L1 therapies to alleviate unnecessary pain and suffering caused by adverse reactions to these therapies in patients unlikely to respond to immune checkpoint therapies. Effective screening techniques have been dampened by the low and heterogeneous levels of PD-1 and PD-L1 expression detectable on tumors and immune cells [16].

Evaluation of the binding and pharmacokinetic properties of immune checkpoint inhibitor antibodies *in vivo* requires the use of complex transgenic or humanized animal models. Due to the innate limitations of many transgenic models, the popularity of humanized animal models has exponentially increased over the last decade. The human peripheral blood lymphocytes-severe combined immunodeficiency (hu-PBL-SCID) model, also known as PBL, is the most readily available and cost-effective humanized murine model [17]. This mouse model is generated through engraftment of human peripheral mononuclear blood cells (hPBMCs) into severally immunodeficient NOD/SCID/IL2 γ c null (NSG) mice. However, engraftment of hPBMCs induces chronic graft-versus-host disease (GvHD) in the mice at 4-6 weeks post-engraftment, which can limit their usefulness in therapy studies [18-20]. For PD-1-based studies, the induction of GvHD leads to T-cell activation and increased PD-1 expression, which provides optimal conditions for immune checkpoint blockade imaging-based studies.

Few studies have investigated the role that molecular imaging may play in allowing researchers to visualize immunotherapy biomarkers [9]. For example, Natarajan *et al.* developed a murine anti-PD-1 tracer for positron emission tomography (PET) imaging of tumor-infiltrating lymphocytes in a murine model of mouse melanoma [21]. While the study revealed that PD-1 was an excellent biomarker for T-cell tracking, the use of a mouse antibody failed to address the clinical scenario and barriers faced in human experiments. In a previous study, our group investigated the pharmacokinetic properties and dosimetry of radiolabeled pembrolizumab in a humanized murine model (22); however, the study was limited to non-tumor-bearing mice and failed to address the imaging of infiltrating T-cells in a humanized murine models of cancer. To improve upon the previous study, we have developed a humanized murine model of lung cancer suitable for imaging the interactions between tumor and cancer cells. In this study, we develop and characterize the anti-PD1 tracer (^{89}Zr -Df-nivolumab) for imaging of PD-1-expressing T-cell infiltrates in a humanized mouse model of lung cancer. In the future, immunoPET imaging of cancer using immunotherapeutic antibodies may allow physicians to monitor the efficacy of immunotherapy intervention, and specifically tracking PD-1-expressing activated T-cells, while also improving patient stratification and possibly predicting autoimmune toxicities.

Materials and Methods

Flow cytometry

This protocol was approved by an institutional review board at the University of Wisconsin – Madison, and all patients consented to the study. Blood samples were collected from patients for isolation of hPBMCs using the Ficoll-Histopaque (GE Healthcare, Little Chalfont, UK) technique by density gradient centrifugation. The cells were washed and stimulated for 18 h with 40 ng/mL phorbol myristate acetate (Sigma-Aldrich, St. Louis, MO, USA) and 1.3 $\mu\text{g/mL}$ ionomycin (MP Biomedicals, Santa Ana, CA, USA). After stimulation of the hPBMCs, cells were stained with nivolumab or the chelator – antibody conjugate (Df-Nivolumab) for 1 h at 4°C. Next, the cells were before washed and stained with AlexaFluor₄₈₈-labeled anti-human IgG (Life Technologies, Carlsbad, CA) for 2 h at room temperature. Also, cells were stained with the primary antibodies CD3-v500, CD4-PE, and

CD8-APC antibodies (BD Biosciences, San Jose, CA, USA) and GhostDye Red-780 (Tonbo Biosciences, San Diego, CA, USA). Cells were washed and resuspended in phosphate buffered saline (PBS) with 3% fetal calf serum, and analyzed on a BD LSRFortessa cytometer.

Chelation and radiolabeling procedures

Nivolumab was obtained commercially from Bristol-Myers Squibb Company (New York, NY, USA). The antibody was reconstituted in sterile PBS before conjugation with p-SCN-Deferoxamine (Df; Macrocyclics, Dallas, TX, USA) through the exposed lysine residues using methods previously described in the literature [22]. After mixing the antibody and chelator at a molar ratio of 1:5, the pH was adjusted to ~8.5 using Na₂CO₃ and the mixture was incubated at room temperature for 2 h. After reacting, the solution was purified via PD-10 columns (size exclusion chromatography) with PBS as the mobile phase. The isotope (⁸⁹Zr) was produced in a GE PETtrace cyclotron by irradiation of natural yttrium foils (250 µm, 99.9%) with 13.8 MeV protons using methods previously described [23]. For radiolabeling, 74-148 MBq of neutralized ⁸⁹Zr-oxalate was added to the antibody-chelator solution at a ratio of 0.25 mg of Df-nivolumab per 37 MBq of ⁸⁹Zr. Before incubating at room temperature for 1 h, the volume was adjusted to 1 mL with HEPES buffer. The radiolabeled antibody was purified by PD-10 columns and injected into mice.

Animal models

All animal studies were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. NOD *scid* gamma (NSG), also known as NOD-*scid* IL2Rgamma^{null} mice, were obtained from the UW-Madison Humanized Mouse Core Service. For generation of the hu-PBL-SCID model (PBL), NSG mice at 3-5 weeks of age were subcutaneously injected with 100 µL (1 × 10⁶ cells) of a 1:1 mixture of A549 cells and Matrigel Matrix Basement Membrane (Corning, Corning, New York, USA) into the lower flank of the mice. Tumors were allowed to reach 1-2 mm in diameter before the mice were reconstituted with 0.5 × 10⁶ hPBMCs via tail-vein injection. The animals were allowed to engraft for 14-16 days before use, ensuring that both PBL and NSG mice of the same age and similar engraftment were used for imaging studies. Engraftment efficiency values were provided by the UW-Madison Humanized Mouse Core Service.

PET imaging and analysis

Mice were injected intravenously with 5 – 10 MBq of the tracer (⁸⁹Zr-Df-nivolumab) prior to PET imaging. For imaging, mice were placed in supine position in the Inveon microPET/microCT rodent model scanner (Siemens Medical Solutions, Erlangen, Germany). Scans were performed with 40-million coincidence events static scans being recorded. Images were reconstructed using the 3D ordered subset expectation maximization algorithm and quantified via region-of-interest (ROI) analysis in the Inveon Research Workplace software (Siemens Medical Solutions). Signal quantification was expressed as the percentage of injected dose per gram of tissue (%ID/g).

Ex vivo biodistribution studies

Mice were euthanized via CO₂ asphyxiation after the final scan for *ex vivo* biodistribution and immunohistochemistry studies. Major tissues, organs, and blood were collected for measurement of radioactivity. Each sample was wet-weighed before the radioactivity was counted using the Perkin Elmer Wizard2 automatic γ -counter (Waltham, MA, USA). Values were normalized and recorded as %ID/g.

Immunohistochemistry

Tumor, salivary gland, spleen, and lung tissue were embedded in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA, USA). Frozen tissues were sectioned at 5- μ m thickness and fixed with ice-cold acetone for 10 min before being rehydrated in PBS for 5 min. Slides were blocked with 10% normal donkey serum in 0.25% Triton-X in PBS for 1 h at room temperature. For primary antibodies, 1:200 dilutions of rabbit anti-human CD3 (Novus Biologicals, Littleton, CO, USA) and goat anti-human PD-1 (Novus Biologicals) were prepared in the blocking solution and incubated with the tissue for 12 h at 4 °C. Slides were washed three times with PBS for 15 min at room temperature. For secondary antibodies, 1:1000 donkey-anti rabbit IgG DyLight 550 (Novus Biologicals) and 1:2000 donkey anti-goat IgG DyLight 488 (Novus Biologicals) were made using the blocking solution. The slides were incubated with secondary antibodies for 1 h at room temperature. Next, slides were washed three times with PBS for 10 min before mounting using a mixture of Fluoromount (Novus Biologicals) and Vectashield with DAPI (Vector Labs, Burlingame, CA, USA). The Nikon A1RS system was used to image slides.

Radiation dosimetry extrapolation to humans

The OLINDA/EXM software was used for dosimetry analysis. The %ID/g values obtained from PET imaging of the tracer in mice were used to estimate human dosimetry. The biodistribution of the tracer was assumed to be the same between humans and mice allowing for the use of a monoexponential model. OLINDA provides effective dose outputs and weighting factors from International Commission on Radiological Protection Publication 103 were employed to convert to absorbed dose in each organ [24].

Statistical analysis

Quantitative data were expressed as the mean $\pm$ standard deviation with all error bars denoting the standard deviation. Means were compared using the Student *t*-test and *p*-values 0.05 were considered statistically significant.

Results

Binding of nivolumab to PD-1 expressed on T-cells

Binding of nivolumab to PD-1 expressed on the surface of stimulated and unstimulated CD4⁺ and CD8⁺ T-cells was assessed via flow cytometry (Fig. 1). PMA-Ionomycin is a potent stimulator of lymphocytes known to quickly upregulate PD-1 expression *in vitro* [25]. Increased binding of nivolumab to both CD4⁺ and CD8⁺ T-cells was detected when the cells were stimulated; however, this interaction was 3 to 4-fold lower for unstimulated cells. To

further demonstrate the specificity, a nonspecific human IgG was used in place of nivolumab. In return, minimal binding was detected between stimulated/unstimulated T-cells and the non-specific human IgG. Also, flow cytometry was used to evaluate if conjugation of the chelator (Df) would inhibit or alter the binding affinity of nivolumab. Both nivolumab and Df-nivolumab showed similar mean fluorescence intensities when incubated with stimulated CD4⁺ and CD8⁺ T-cells, indicating that the chelation and radiolabeling of nivolumab would not affect its binding affinity *in vivo*.

Chelation and radiolabeling of nivolumab

Nivolumab was conjugated with the chelator (Df) for radiolabeling with ⁸⁹Zr. The purified radiolabeled antibody displayed a specific activity of ~ 700 mBq per mg antibody. The radiolabeling efficiency was high with more than 85% of the isotope being chelated after 1 h of incubation, as determined by instant thin-layer chromatography.

PET imaging and biodistribution of ⁸⁹Zr-Df-nivolumab in humanized tumor-bearing mice

After purification, ⁸⁹Zr-Df-nivolumab was injected and longitudinal PET imaging studies were performed at 3, 6, 12, 24, 48, 72, and 168 h post-injection. Maximum intensity projections were acquired for data analysis. Initial imaging time points revealed similar pharmacokinetic properties of the tracer in NSG and PBL mice (Fig. 2). Differences in the biodistribution profile of the tracer became visible by 24 h post-injection. Quantification of PET data via ROI analysis allowed for comparison between tracer uptake in various organs and tissues, including the tumor, salivary glands, blood, liver, spleen, kidney, and muscle tissues. In both PBL and NSG mice, tumors were identified on the left flank by 6 h post-injection. While tumor uptake in PBL mice continued to increase gradually throughout the study, uptake in NSG mice was significantly lower, reached maximum values by 24 h post-injection, and slowly declined by later time points.

As suggested by the maximum intensity projection images (Fig. 3), tumor accumulation was similar between NSG and PBL mice initially; however, PBL mice displayed higher uptake starting at 72 h after injection and onward (n=4, p<0.05; Fig. 3a). More specifically, tumor uptake values of 9.85 ± 2.73 and 3.88 ± 0.38 were obtained 168 h post-injection of ⁸⁹Zr-Df-nivolumab into PBL and NSG mice, respectively (Table S1). In a previous study, our group verified that development of GvHD results in infiltration and permanent residence of lymphocytes into the lacrimal and salivary glands [22]. As expected, similar results were found in this study with PBL mice displaying significantly higher salivary gland uptake of ⁸⁹Zr-Df-nivolumab in comparison to NSG mice (n=4, p<0.05; Fig. 3b). By 168 h post-injection, there was a 2-fold difference in salivary gland signal between PBL (8.38 ± 0.98 %ID/g) and NSG mice (3.30 ± 0.22 %ID/g), further validating the binding specificity of our tracer to its native receptor found on the surface of activated T-cells localized in the tumor and salivary glands.

The tracer showed similar characteristics in reference to blood circulation and off-target accumulation in both PBL and NSG mice. For PBL mice, tracer uptake in the blood pool was 15.5 ± 1.29 %ID/g and 2.70 ± 0.42 %ID/g at the initial and final time points, respectively (Fig. 3c-d). Similarly, blood pool uptake in NSG mice was 13.1 ± 0.79 %ID/g

and 1.88 ± 0.10 %ID/g ($n=4$) at the same time points, suggesting that the tracer remained relatively stable in circulation. Tracer uptake in the kidney and muscle tissues was similar between both groups of mice. In the liver, NSG mice showed higher accumulation likely due to off-target accumulation and antibody metabolism. After the final imaging session, select organs and tissues were extracted for further biodistribution evaluation by *ex vivo* studies. Similar to PET findings, *ex vivo* biodistribution studies revealed key differences between tumor and salivary gland uptake, which were 2 to 3-fold higher in PBL mice (Fig. 4). Additionally, bone and spleen uptake was higher in NSG mice, which was not clearly represented by PET imaging. These differences were attributed to spleen size differences and large biases introduced by the partial volume effect [26].

To verify the specificity of the tracer, PBL mice were injected with a nonspecific tracer (^{89}Zr -Df-IgG) for imaging (Fig. 5). Imaging studies revealed lower tumor and salivary gland uptake in PBL mice injected with the nonspecific tracer in comparison to ^{89}Zr -Df-nivolumab. For example, tumor signal was significantly lower in PBL mice injected with the nonspecific tracer as compared to ^{89}Zr -Df-nivolumab at 3 h post-injection (0.74 ± 0.32 versus 2.46 ± 0.26 %ID/g) and all time points thereafter ($n=4$, $p<0.05$; Fig. 6a). At the final time point (168 h), ^{89}Zr -Df-nivolumab uptake in the tumors was 3-fold higher than ^{89}Zr -Df-IgG, with values of 9.85 ± 2.73 %ID/g and 2.85 ± 0.39 %ID/g, respectively. In respect to the salivary gland uptake, there was a 2-fold difference between the two tracers with ^{89}Zr -Df-nivolumab (8.38 ± 0.98 %ID/g) being significantly higher than ^{89}Zr -Df-IgG (4.65 ± 0.39 %ID/g) at 168 h after injection ($n=4$, $p<0.05$; Fig. 6b). An increase in liver uptake was noticed with the nonspecific group; however, this is a common occurrence that naturally occurs with nonspecific antibodies, yet the cause of this phenomenon remains to be elucidated. Time activity curves show that blood pool uptake steadily decreased after injection, while spleen and muscle accumulation remained steady throughout the study. As demonstrated by PET imaging, the high liver uptake observed in mice injected with the nonspecific tracer was verified by *ex vivo* biodistribution studies (Fig. 6c-d).

Verification of PD-1 expression by immunohistochemistry

Immunofluorescence imaging was used to further validate the imaging findings (Fig. 7). An anti-cluster of differentiation (CD3) antibody was used as a T-cell marker as shown in green, while PD-1 expression was signified by the red color. PD-1 expression was detected in the tumor, salivary gland, spleen, and lung sections of PBL mice, with the strongest signal being in the tumor and spleen. CD3 signal was also detectable in the four tissue sections with the tumor showing the highest degree of CD3. The nuclei were stained blue to verify the presence of cells. In the NSG tissue sections, minimal PD-1 and CD3 expression were visible.

Radiation dosimetry

Dosimetry studies were performed to extrapolate the doses to mice following injection of ^{89}Zr -Df-nivolumab or ^{89}Zr -Df-IgG to adult human females (Table 1). Notable differences in dose were observed for the liver and salivary glands across groups, with other organs providing uniform results. In PBL mice, higher doses to the liver were found with the nonspecific antibody with values of 1.71 ± 0.11 mGy/MBq and 0.98 ± 0.12 mGy/MBq

for ^{89}Zr -Df-IgG and ^{89}Zr -Df-nivolumab, respectively. This was attributed to the rapid clearance of nivolumab from circulation when no antigens for attachment were detected in circulation. In addition, notable doses to the salivary glands in all PBL mice (1.61 ± 0.15 mGy/MBq in Nivolumab PBL and 1.0 ± 0.15 mGy/MBq in IgG PBL) were the result of the GvHD process. As shown, low doses to tumors were observed across all groups. For example, doses of 0.025 ± 0.003 mGy/MBq for radiolabeled nivolumab in PBL mice, 0.020 ± 0.002 mGy/MBq for radiolabeled nivolumab in NSG mice, and 0.013 ± 0.001 mGy/MBq for radiolabeled nonspecific IgG in PBL mice, reported for a tumor mass of 2 g. Even considering these differences, similar total body effective doses were calculated across all groups to be about 0.3 mSv/MBq.

Discussion

Despite being the most preventable malignancy, lung cancer remains the leading cause of cancer-related death worldwide, accounting for nearly 1.6 million estimated deaths in 2017 [27]. Detection of early disease remains critical to successful treatment, yet the lack of physical symptoms results in more than 70% of patients presenting with advanced stage disease (stage III/IV) at the time of diagnosis. While surgical intervention yields the highest cure rates, most patients with advanced stage disease are not candidates for surgery. Hence, these patients rely on radiation therapy, chemotherapy, and newer targeted therapies like immune checkpoint inhibitors. With this in mind, the newly approved immunotherapy agents pembrolizumab and nivolumab have significantly improved the survival rates in a subset of lung cancer patients [28].

In this study, we demonstrated that T-cells expressing PD-1 could be effectively visualized using our novel PD-1 tracer, ^{89}Zr -Df-nivolumab. While most imaging agents are used to directly target the tissue of interest, this work explores the utilization of an imaging agent for non-directly imaging tumor cells. Previously, Natarajan *et al.* demonstrated the specificity of a murine PD-1 tracer for imaging PD-1-expressing tumor infiltrating lymphocytes using ^{64}Cu in a transgenic mouse model of melanoma [29]. Tumor uptake was 7.4 ± 0.71 %ID/g at the final imaging time point (48 h post-injection), which was lower than the tumor accumulation found in this study (9.85 ± 2.73 %ID/g). However, our value was obtained at 168 h post-injection, suggesting that the humanized antibody requires more time to accumulate in the tumor as compared to the murine antibody. Additionally, the murine tracer may have continued to accumulate if the study was not halted at 48 h post-injection; however, the short half-life of ^{64}Cu (12 h) was a limiting factor. By employing the long decay half-life properties of ^{89}Zr (3.3 days), this study allowed for the tracking of PD-1-expressing T-cells infiltration into the tumor over the course of 168 h (Fig. 2). In addition, it is important to note that tumor uptake is less than that usually noted with anti-tumor antibodies; however, this is because our imaging agent primarily targets T-cells localized in the tumor (non-direct imaging) and not the actual tumor cells.

Despite an increase in the popularity of immunotherapies, limited studies have characterized the biodistribution and pharmacokinetic properties of immune checkpoint inhibitor antibodies *in vivo* outside of clinical trials. In a previous study, England *et al.* demonstrated for the first time that radiolabeled pembrolizumab showed a unique biodistribution profile in

humanized mice injected with hPBMCs when compared to non-humanized mice. Radiolabeled pembrolizumab was shown to accumulate in the salivary glands of PBL mice. Similarly, this study revealed increased salivary gland uptake of the tracer (Fig. 3), which has been attributed to T-cells trafficking to the salivary glands as a result of GvHD and other autoimmune diseases [30]. Another interesting finding in this study was the increased spleen uptake of the tracer found in the NSG model (Fig. 2). This is, in part, the result of spleen size as the spleens of NSG mice are significantly smaller than those of immunocompetent mice. Additionally, the spleens of PBL mice can enlarge by 10-50 once injected with hPBMCs [31]; hence, spleen uptake was higher in NSG mice because the smaller spleen led to a large bias introduced by the partial volume effect. Unlike our previous investigation of pembrolizumab, PBL and NSG mice were implanted with tumor cells in this study to assess if targeting T-cells would allow for tumor imaging.

This study demonstrated that humanized murine models of cancer may play important roles in the study and development of future immunotherapy agents. In this study, tracer accumulation is attributed to T-cell infiltration into the tumor, yet tumor cells do not display PD-1 expression. The process of tumor infiltration by lymphocytes has been extensively documented and this type of study may provide additional data regarding the process [32]. Using PBL mice injected with ^{89}Zr -Df-IgG, we verified that the accumulation of our novel tracer was highly specific and not due to off-target accumulation (Fig. 6). In the future, the use of more complex humanized models of cancer may provide added benefits. For example, a limitation of this study was the innate variability caused by the lack of a stable model, as the xenograft efficiency varied between mice. Despite ensuring that all mice met the minimum xenograft efficiency required for study inclusion, the slight variations in xenograft efficiency and GvHD severity could have resulted in small differences we found in mice of the same group.

There are many potential applications likely to benefit from the PD-1 imaging agents. As described earlier, PD-1-based imaging agents may allow physicians to determine which patients are more apt to benefit from anti-PD-1 or anti-PD-L1 therapies in the future. In return, this could save thousands of patients from the unnecessary adverse effects associated with immune checkpoint therapies. Also, PD-1 imaging may be utilized to track T-cells that have become activated by various therapies to gain insight into the interactions between T-cell activation, homing, and tumor residence. These new imaging agents may play an essential role in assisting researchers and physicians determine other potential toxicities that may be associated with newer anti-PD-1 therapies in the future. A limitation for use of immune checkpoint antibodies as imaging agents is that immune checkpoint pathway receptors are heterogeneously expressed in multiple areas of the body, which complicates potential imaging strategies. Hence, innovative approaches are required to target many immunotherapy biomarkers, which create a key limitation in the clinical utility of these agents at this time for imaging-related studies. Lastly, the presence of PD-1 on the surface of tumor cells may also limit the potential of PD-1-targeted imaging agents as a predictive or pharmacodynamics biomarker as this could bias our judgment on treatment prediction. However, PD-1 expression on the surface of T-cells is much higher than that of any tumor cells, suggesting that PD-1 expression from tumor cells would have minimal effect on imaging studies.

Conclusions

Herein, this study demonstrates that radiolabeled nivolumab may be used to noninvasively image PD-1 expression in humanized tumor-bearing mice. More specifically, the infiltration of PD-1-expressing T-cells was effectively imaged in a humanized mouse model implanted with A549 tumors. As the field of cancer immunotherapy is expected to undergo rapid growth during the next decade, improved strategies like noninvasive PET are valuable tools for investigating the biodistribution of newly developed antibody therapies. In the future, imaging of immunotherapy biomarkers may be used for therapeutic monitoring and patient stratification.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

Cell binding assay of nivolumab to unstimulated or stimulated CD4⁺ and CD8⁺ T-cells. The T-cells were stimulated to express PD-1 before incubation with IgG, nivolumab, or Df-nivolumab. Similar binding was displayed by nivolumab and Df-nivolumab indicating that binding was unaffected by chelation of the antibody. Nivolumab revealed significantly higher binding to stimulated T-cells, and minimal binding was detected with the nonspecific antibody.

Fig. 2. PET imaging of ^{89}Zr -Df-nivolumab in PBL and NSG mice. Maximum intensity projections are shown from 3 to 168 h post-injection of the tracer with the scale ranging from 0 to 10 %ID/g.

Fig. 3.

Analysis of PET imaging of ^{89}Zr -Df-nivolumab in NSG and PBL mice. (a) Tumor uptake of ^{89}Zr -Df-nivolumab was quantified and revealed significant differences after 72 h post-injection ($n=4$, $p = 0.05$). (b) Salivary gland uptake of ^{89}Zr -Df-nivolumab was quantified and revealed significant differences after 24 h post-injection ($n=4$, $p = 0.05$). (c/d) PET region-of-interest analysis as time-activity curves of the signal in the blood, liver, spleen, kidneys, and muscle after intravenous injection of ^{89}Zr -Df-nivolumab in (c) NSG and (d) PBL mice ($n=4$). SG - salivary gland.

Fig. 4.

Ex vivo biodistribution of ^{89}Zr -Df-nivolumab in NSG and PBL mice at 168 h post-injection (n=4). SG - salivary gland.

Fig. 5. PET imaging of ^{89}Zr -Df-IgG in PBL mice. Maximum intensity projections are shown from 3 to 168 h post-injection of the tracer with the scale ranging from 0 to 10 %ID/g. SG - salivary gland.

Fig. 6.

Analysis of PET imaging of $^{89}\text{Zr-Df-IgG}$ in PBL mice. (a) Tumor uptake of $^{89}\text{Zr-Df-IgG}$ in PBL mice was quantified and showed significant differences from the first time point onward from PBL mice injected with $^{89}\text{Zr-Df-nivolumab}$ ($n=4$, $p < 0.05$). (b) Salivary gland uptake of $^{89}\text{Zr-Df-IgG}$ was quantified and showed significant differences from PBL mice injected with $^{89}\text{Zr-Df-nivolumab}$ starting at 6 h post-injection ($n=4$, $p < 0.05$). (c) PET region-of-interest analysis as time-activity curves of the signal in the blood, liver, spleen, kidneys, and muscle after intravenous injection of $^{89}\text{Zr-Df-IgG}$. (d) *Ex vivo* biodistribution of $^{89}\text{Zr-Df-IgG}$ in PBL mice. SG – Salivary gland.

Fig. 7. Histological staining of tumor, salivary gland, spleen, and lung tissue sections from (a) PBL and (b) NSG mice to verify the localization of PD-1 and CD3⁺ (T-cell marker). DAPI: 4',6-diamidino-2-phenylindole, Scale bar: 20 μ m.

Estimated radiation absorbed doses to an adult human after intravenous injection of ^{89}Zr -Df-nivolumab in PBL and NSG mice, along with ^{89}Zr -Df-IgG in PBL mice (n = 4). SD – standard deviation.

Table 1

Organ	^{89}Zr -Df-nivolumab in NSG (mGy/MBq)		^{89}Zr -Df-nivolumab in PBL (mGy/MBq)		^{89}Zr -Df-IgG in PBL (mGy/MBq)	
	Average	SD	Average	SD	Average	SD
Adrenals	0.013	0.001	0.012	0.001	0.015	0.000
Brain	0.064	0.005	0.058	0.005	0.055	0.002
Breasts	0.059	0.004	0.056	0.004	0.059	0.001
Small Intestines	0.008	0.001	0.008	0.001	0.008	0.000
Stomach Wall	0.231	0.017	0.211	0.017	0.231	0.003
Kidneys	0.026	0.004	0.020	0.002	0.024	0.001
Liver	0.973	0.077	0.983	0.121	1.708	0.112
Lungs	0.209	0.014	0.200	0.016	0.228	0.003
Muscle	0.007	0.001	0.007	0.001	0.007	0.000
Ovaries	0.495	0.037	0.452	0.036	0.448	0.013
Pancreas	0.013	0.001	0.012	0.001	0.014	0.001
Red Marrow	0.177	0.013	0.164	0.013	0.171	0.003
Skin	0.119	0.009	0.111	0.009	0.113	0.003
Spleen	0.028	0.002	0.021	0.001	0.018	0.001
Thymus	0.008	0.001	0.008	0.001	0.008	0.000
Salivary Glands	0.844	0.036	1.605	0.154	0.999	0.150
Urinary Bladder	0.180	0.014	0.164	0.013	0.160	0.005
Uterus	0.008	0.001	0.007	0.001	0.007	0.000
Effective Dose (mGy/MBq)	0.387	0.032	0.309	0.019	0.351	0.018