⁸⁹Zr-Labeled Anti-PD-L1 Antibody Fragment for Evaluating *In Vivo* PD-L1 Levels in Melanoma Mouse Model

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Abstract

The rise of programmed death-1 (PD-1)/PD-L1 immune checkpoint inhibitor therapy has been one of the most promising developments in melanoma research. However, not all the melanoma patients respond to such immune checkpoint blockade. There is a great need of biomarkers for appropriate melanoma patient selection and therapeutic efficacy monitoring. The objective of this study is to develop a novel radiolabeled anti-PD-L1 antibody fragment, as an imaging biomarker, for evaluating the *in vivo* PD-L1 levels in melanoma. The Df-conjugated F(ab')₂ fragment of the anti-mouse PD-L1 antibody was successfully synthesized and radiolabeled with ⁸⁹Zr. Both Df-F(ab')₂ and ⁸⁹Zr-Df-F(ab')₂ maintained the nano-molar murine PD-L1 targeting specificity and affinity. ⁸⁹Zr-Df-F(ab')₂ showed less uptake in normal liver tissue in mice compared with its full antibody counterpart ⁸⁹Zr-Df-anti-PD-L1. Positron emission tomography (PET)/computed tomography images clearly showed that ⁸⁹Zr-Df-F(ab')₂ possessed superior pharmacokinetics and imaging contrast over the radiolabeled full antibody, with much earlier and higher tumor uptake (5.5 times more at 2 h post injection) and much lower liver background (51% reduction at 2 h post injection). The specific and high murine PD-L1-targeting uptake at tumor foci coupled with fast clearance of ⁸⁹Zr-Df-F(ab')₂ highlighted its potential for *in vivo* PET imaging of murine PD-L1 levels and future development of radiolabeled anti-human PD-L1 fragment for potential application in melanoma patients.

Keywords: antibody fragment, immune checkpoint inhibitor therapy, melanoma, PD-L1 biomarker, PET imaging

Introduction

RECENTLY, THE RISE of immunotherapy, especially the immune checkpoint inhibitor therapy, has been one of the most promising developments in melanoma research.^{1,2} Inside the melanoma microenvironment, the programmed death-1 (PD-1) receptor is expressed on activated T lymphocytes, and will trigger inhibitory signal into the T lymphocytes upon binding to its ligand PD-L1.^{3,4} At the meantime, PD-L1 expressed on melanoma cell surface, has been upregulated by melanoma to escape the T lymphocytes' immune recognition and destruction. In recent research, antibody inhibitors, which block the PD-1/PD-L1 pathway, has been very successful in re-activating T lymphocytes to find and destroy melanoma cells. Furthermore, FDA-approved

anti-PD-1 and PD-L1 antibodies have showed impressive response in patients with melanoma.^{2,5,6}

Unfortunately, not all the melanoma patients respond to PD-1/PD-L1 immune checkpoint blockade. Despite impressive treatment outcomes in a subset of melanoma patients (10%–40% with monotherapy), many patients fail to respond to immune checkpoint inhibitor therapies.⁷ Many challenges exist, including appropriate patient selection and therapeutic efficacy monitoring.⁸ Such screening biomarker is urgently needed to ensure those responding patients receive timely immune checkpoint inhibitor treatment, and at the same time not to delay the remaining patients for other treatment options. Additionally, during the treatment, the cancer will evolve and some of the patients might not be suitable for further treatment anymore. Hence, there is also

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an urgent need of biomarkers to monitor and re-evaluate the cancer patients to determine whether they are still responding to the immune checkpoint inhibitor treatment.

Currently, the general method to collect patient information for such immunotherapy is to use the invasive tissue biopsy, which will take a small portion of the cancer tissue from the patients for immunohistochemical staining.^{9,10} Unfortunately, there are multiple limitations of such biopsy testing. First, the sample taken out of the patients cannot represent the patient's real time status as it will be tested at a later time. Furthermore, the sample is fixed and will lose valuable information for diagnosis. Second, as a tumor develops, it evolves and creates a complex microenvironment. Hence, the small biopsy sample may not represent the detailed information of the whole tumor, especially when there is remote tumor metastasis occurring. Biopsy is also faced with many other drawbacks, including its invasive nature, procedure-associated risks, sampling variability, and limited spatial information.

Molecular imaging, especially positron emission tomography (PET), provides a sensitive and quantitative tool to noninvasively assess targets inside the body and monitor therapeutic response. In contrast to biopsy, PET imaging is able to quantitatively and longitudinally monitor the immune checkpoint PD-L1 levels in the whole tumor lesions and metastases, which avoids sampling errors and misinterpretation caused by the intra-tumoral and inter-lesional heterogeneity.¹¹ Hence, PET imaging could potentially serve as a predictive biomarker for selecting patients for anti-PD-1/PD-L1 immune checkpoint inhibitor therapy. Furthermore, it also holds potential for the longitudinal assessment of therapy responses.

Recently, radiolabeled anti-PD-L1 antibody has been developed and evaluated in tumor animal models or patients for *in vivo* imaging of PD-L1 levels.^{12–16} Specifically, ⁸⁹Zr-atezolizumab (anti-human PD-L1) and ¹⁸F-labeled adnectin have been used for human cancer patients PET imaging and successfully visualized the PD-L1 levels in tumor foci.^{17,18} With the specific target (PD-L1) and ligand (anti-PD-L1 antibody) recognition, such radiolabeled antibody derivatives could specifically accumulate at the PD-L1-positive tumor foci and visualize the PD-L1 levels via PET signals. However, the major drawback of such antibody tracers for imaging has been their high liver accumulation and prolonged circulating half-life. Because of such drawbacks, the tumor uptake typically does not reach its peak until a few days post tracer injection. On the other hand, the bioactive fragment of the whole antibody possesses much lower normal organ (especially the liver) accumulation than its corresponding intact whole antibody.^{19–21} More importantly, the bioactive fragment has a much shorter *in vivo* half-life (hours) than the whole antibody (days), which will enable us to potentially use such fragment imaging biomarkers daily to monitor the real-time expression level of PD-L1 in melanoma animal models or patients.

The goal of this study is to prepare and investigate the *in vitro* and *in vivo* characteristics of a ⁸⁹Zr-labeled bioactive fragment of the mouse anti-PD-L1 antibody (10F.9G2 clone) for PET imaging of PD-L1 levels in a B16F10 murine melanoma model. In this study, we synthesized the radiolabeled fragment ⁸⁹Zr-Df-F(ab')₂. The PD-L1-targeting specificity and affinity of ⁸⁹Zr-Df-F(ab')₂ was determined in

PD-L1-positive murine melanoma B16F10 cells. The pharmacokinetics of ⁸⁹Zr-Df-F(ab')₂ was determined in wild-type C57 mice and compared with its radiolabeled full antibody counterpart. PET imaging characteristics of ⁸⁹Zr-Df-F(ab')₂ was evaluated in B16F10 flank tumor-bearing mice. Flow cytometry was employed for post imaging analysis of the tumor and spleen samples.

Materials and Methods

Chemicals and reagents

Antibodies (InVivoMab anti-mouse PD-L1 [B7-H1], 10F.9G2 clone and InVivoMab rat IgG2b isotype control, LTF-2 clone) were purchased from BioXCell (West Lebanon, NH). *p*-SCN-Bn-Deferoxamine (Df) was purchased from Macrocyclics (Plano, TX). ⁸⁹Zr oxalate was purchased from the Radionuclide Processing Laboratory (RPL) of Mallinckrodt Institute of Radiology at Washington University at St. Louis. Zeba™ Spin desalting columns were purchased from ThermoFisher Scientific (Waltham, MA). Illustra NAP-10 disposable column was from GE Life Sciences. APC-Cy7-viability dye, APC-anti-PD-L1-antibody, APC-IgG2b, and other antibodies were purchased from BioLegend. All the other chemicals and reagents were purchased from Sigma-Aldrich and used without further purification. B16F10 murine melanoma cell lines were obtained from American Type Culture Collection (Manassas, VA), and C57BL/6 mice were purchased from Charles River Laboratories.

Preparation of F(ab')₂ fragment

F(ab')₂ fragment of anti-PD-L1 antibody (10F.9G2 clone) was prepared according to the literature with some modifications.^{19,21,22} Briefly, InVivoMab anti-mouse PD-L1 antibody (4.5 mg) in 0.5 M NH₄OAc buffer (pH 3.68) was mixed with freshly prepared pepsin solution (1 mg/mL in 10 mM HCl aqueous solution) and 300 μL of 0.5 M NH₄OAc buffer at 37°C for 4 h. At the end of the reaction, the reaction mixture was loaded onto a GE AKTA_{prime} plus FPLC system for purification. The GE Hiload 16/600 Superdex 75pg size exclusion column (SEC), ultraviolet (UV) 280 nm filter, and a flowrate of 1 mL/min 1×phosphate buffered saline (PBS) (with 0.025% NaN₃) were used to separate the F(ab')₂ fragment from the Fc, pepsin, and other digested components in the reaction mixture. Aliquot of 0.5 mL of eluent was collected into 5 mL plastic tube with a fraction collector, and tubes for the same peak were combined. The purity of collected peaks were verified with both GE AKTA_{prime} plus and Bio-Rad Dualflow (with a Phenomenex Yarra 3 μm SEC-2000 column) FPLC systems. The purified F(ab')₂ was then characterized by MALDI-TOF mass spectrometry.

Nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis characterization of F(ab')₂ fragment

Ten microliters of standard protein ladder solution (Bio-Rad Precision Plus Protein Dual Color Standards), anti-PD-L1 antibody stock solution (10F.9G2 clone; BioXCell), or the collected peaks from FPLC was mixed with equivalent volume of Bio-Rad 2×Laemmli sample buffer in 1.7 mL Eppendorf tubes, and then heated at 100°C on a VWR heating block for 10 min. The mixture was then cooled

down to room temperature using ice, and loaded onto a Bio-Rad 10% MP TGX Stain-Free Gel and run with a Mini-Protein Tetra system (200 keV for 35 min; 1×Tris/glycine/SDS buffer). The gel was then stained with Coomassie G-250 for scan and analysis.

Synthesis of $^{89}\text{Zr-Df-F(ab')}_2$ and $^{89}\text{Zr-Df-anti-PD-L1}$

FPLC-purified F(ab')_2 fragment of anti-PD-L1 antibody (10F.9G2 clone, 0.65 mg in 1 mL 1×PBS) or stock anti-PD-L1 antibody (1 mg in 1×PBS) was passed through the Zeba spin desalting column (7k MWCO, 2 mL) and switched buffer to 0.1 M sodium carbonate (pH 9.4). Then, 20 μL of freshly prepared *p*-SCN-Bn-Deferoxamine solution (10 mg/mL in DMSO, Df-to-protein molar ratio = 40:1) was slowly added to the protein solution. Another 100 μL of sodium carbonate buffer (0.1 M, pH 9.0) was added, and the reaction mixture was put on a rotatory shaker for gentle shaking at room temperature for 2 h in the dark. Df-F(ab')_2 or Df-anti-PD-L1 was then purified by passing the reaction mixture through two Zeba spin desalting columns (7k MWCO, 2 mL) and two NAP-10 columns. Every 0.5 mL of the eluent from the last NAP-10 column was collected in each tube, and tested with NanoDrop UV-Vis Spectrophotometers for peak selection and protein concentration determination. The purified Df-F(ab')_2 was then characterized by MALDI-TOF mass spectrometry.

To prepare the radiolabeled proteins, 100 μg of the Df-F(ab')_2 or Df-anti-PD-L1 in 1×PBS solution was added to 60 μL of ^{89}Zr oxalate solution (60 MBq, already neutralized with 0.5 M sodium carbonate buffer) and mixed with 100 μL of 0.5 M HEPES buffer (pH 7.5). Then, the reaction mixture was incubated at 37°C for 1 h, followed by a NAP-10 column purification to collect the pure $^{89}\text{Zr-Df-F(ab')}_2$ or $^{89}\text{Zr-Df-anti-PD-L1}$. *In vitro* stabilities of $^{89}\text{Zr-Df-F(ab')}_2$ and $^{89}\text{Zr-Df-anti-PD-L1}$ were determined with NAP-10 column daily for up to 1 week by storing the radiolabeled proteins in a 4°C refrigerator. Stability at 37°C with and without the presence of mouse serum was evaluated as well.

Flow cytometry verification of PD-L1 expression on B16F10 cells

B16F10 cells were cultured in RPMI-1640 medium (10% fetal bovine serum plus 1% penicillin and streptomycin) and harvested for flow cytometry analysis when reaching an 80% confluence. Two million suspended B16F10 cells were first placed into a 5 mL polystyrene tube and incubated for 10 min with 2 μL of Fc blocking reagent (anti-CD16/CD32) at 4°C. The APC-Cy7-viability dye (1 μL of the prediluted solution from BioLegend for 1 million cells) and APC-anti-PD-L1 (BioLegend, 1 μL 0.2 mg/mL stock for 1 million cells) was then added to the cells, and the mixture was incubated at 4°C for 30 min. APC-IgG2b was used as the isotype control. Flow cytometry analysis was then conducted with a BD FACSCanto II, and the histogram and mean fluorescent intensity (MFI) of PD-L1 expression on B16F10 cells was determined.

In vitro competitive binding assay

The receptor-binding affinities (inhibitory concentration of 50% [IC_{50}]) of F(ab')_2 and Df-F(ab')_2 were determined by *in vitro* competitive binding assay according to our

previously published procedure.^{23,24} Briefly, B16F10 cells were harvested from culture flask at 80% confluence and seeded into a 24-well cell culture plate (2×10^5 cells/well) and incubated at 37°C overnight. After washing twice with binding medium (RPMI-1640), the cells were incubated at room temperature (25°C) for 30 min with $\sim 50,000$ cpm of $^{89}\text{Zr-Df-anti-PD-L1}$ in the presence of increasing concentrations (10^{-13} to 10^{-6} M) of protein in 0.3 mL of binding medium. The reaction medium was aspirated after the incubation. The cells were then rinsed three times with 0.5 mL of ice-cold PBS (1×PBS, pH 7.4, 0.2% bovine serum albumin [BSA]) and lysed in 0.5 mL of 1 N NaOH for 5 min. The radioactivity associated with cells was measured in a 2470 Wizard² automatic gamma counter (PerkinElmer, NJ). The IC_{50} values were calculated using the Prism software (GraphPad Software, La Jolla, CA).

In vitro B16F10 cell binding assay

The PD-L1 specificity of $^{89}\text{Zr-Df-F(ab')}_2$ was determined by *in vitro* cell binding assay according to our previously published procedure.²⁵ Briefly, B16F10 cells were harvested and seeded into a 24-well cell culture plate (2×10^5 cells/well) and incubated at 37°C overnight. After washing twice with binding medium (RPMI-1640), the cells were incubated at room temperature (25°C) for 0.5 h with $\sim 30,000$ cpm of $^{89}\text{Zr-Df-F(ab')}_2$ in 0.3 mL of binding medium. For anti-PD-L1 antibody and isotype IgG2b blockage, the final blocking concentration was adjusted to 1 μM . The reaction medium was aspirated after the incubation and cells were rinsed three times with 0.5 mL of ice-cold PBS (1×, pH 7.4, 0.2% BSA) and lysed in 0.5 mL of 1 N NaOH for 5 min. The radioactivity associated with cells was measured with a gamma counter.

Flow cytometry analysis of fluorescein isothiocyanate-tagged antibody and its fragment on B16F10 cells

Fluorescein isothiocyanate (FITC)-anti-PD-L1 (10F.9G2 clone), FITC- F(ab')_2 , and FITC-isotype IgG2b were prepared using the same reacting conditions used for Df-conjugation in this study (pH 9.0, 2 h incubation at RT, SEC purification). For cell staining, two million suspended B16F10 cells were first placed into a 5 mL polystyrene tube and incubated for 10 min with 2 μL of Fc blocking reagent (anti-CD16/CD32) at 4°C. FITC-anti-PD-L1, FITC- F(ab')_2 , or FITC-isotype IgG2b was then added to the cells, and the mixture was incubated at 4°C for 30 min. Flow cytometry analysis was then conducted with a BD FACSCanto II, and the histogram and MFI of each protein on B16F10 cells were determined.

Biodistribution studies

All the animal studies were conducted in compliance with the Institutional Animal Care and Use Committee approval at the University of Louisville. The mice were housed five animals per cage in sterile micro-isolator cages in a temperature- and humidity-controlled room with a 12 h light/12 h dark schedule. The biodistribution of $^{89}\text{Zr-Df-F(ab')}_2$ was determined in C57 female mice (Charles River) according to our previously published procedure.²⁴ Briefly, 0.037 MBq (0.1 μg of protein) of $^{89}\text{Zr-Df-F(ab')}_2$ was injected via tail vein for each mouse. Groups of five mice were

euthanized at 0.5, 4, and 24 h post injection, and organs of interest were harvested, weighed, and counted. Biodistribution of ^{89}Zr -Df-anti-PD-L1 was also determined in C57 mice at 4 and 24 h post injection for comparison.

PET/computed tomography imaging with ^{89}Zr -Df-F(ab')₂ and ^{89}Zr -Df-anti-PD-L1

PET/computed tomography (CT) imaging was conducted in B16F10 flank melanoma mouse model according to our published procedures.²⁴ Briefly, C57 mice were subcutaneously inoculated with 1×10^6 B16F10 cells on the right flank. The mice are ready for imaging when the weight of the tumors reached ~ 0.2 g on 10 d post cell inoculation. Each melanoma-bearing mouse was injected with 6.5 MBq (17.6 μg of protein) of ^{89}Zr -Df-F(ab')₂ or ^{89}Zr -Df-anti-PD-L1 via the tail vein. The mice were scanned for 15 min with Siemens R4 MicroPET and followed by 10 min of CT scan at 2, 24, and 48 h post dose injection. Siemens IAW software was used for the acquisition and reconstruction of the PET signal, and Siemens IRW software was used for merging and analyzing the imaging data. At the end of the imaging study, mice were euthanized, and organs of interest were harvested, weighed, and counted.

Post imaging flow cytometry analysis

Post PET/CT imaging, the mice were euthanized, and tumor and spleen from each mouse were harvested for flow cytometry analysis. Briefly, the *ex vivo* tumor was cut into small pieces and digested with tumor digestion buffer (300 U/mL collagenase I, 60 U/mL hyaluronidase, and 80 U/mL DNase I diluted in RPMI-1640 at a 1:10 dilution for 30 min while being agitated at 37°C). Spleen was smashed with the end of a 3 mL syringe, and both the tumor and spleen were then passed through a 70- μm filter. Two million cells of either tumor or spleen were then placed into sterile 5 mL polystyrene tubes and incubated at 4°C for 10 min with 2 μL of Fc block agent (anti-CD16/CD32). APC-Cy7-viability dye, APC-anti-PD-L1, PerCP-Cy5-anti-CD45, PE-anti-f4/80, and PE-Cy7-anti-CD11b antibodies were then added to cells and incubated at 4°C for 30 min. APC-IgG2b was used as the isotype for PD-L1 staining. Flow cytometry analysis was conducted with BD FACSCanto II, and the histogram and MFI of PD-L1 expression on subsets of cells in both tumor and spleen was determined.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test for unpaired data. A 95% confidence level was chosen to determine the significance between the cell and organ uptakes of ^{89}Zr -Df-F(ab')₂ and ^{89}Zr -Df-anti-PD-L1 in the *in vitro* cell binding and *in vivo* biodistribution and imaging

studies described above. Difference at the 95% confidence level ($p < 0.05$) were considered significant.

Results and Discussion

The goal of this study is to develop novel radiolabeled bioactive fragments of anti-PD-L1 antibody for *in vivo* evaluation of PD-L1 levels with PET imaging. Hence, as showed in Figure 1, the first step of this study is to prepare the F(ab')₂ fragment from the full anti-PD-L1 antibody. This was accomplished by digesting the full antibody with the enzyme pepsin.²¹ The digesting conditions, such as antibody-to-pepsin ratio, reacting pH, reacting temperature, and reacting time, were all tested and the optimized conditions were used in this study to successfully prepare the F(ab')₂ fragment of anti-mouse PD-L1 (B7-H1) antibody by enzymatic cleavage of the Fc portion from the full antibody with pepsin. The resulting F(ab')₂ fragment was purified via size exclusion FPLC from the pepsin, Fc, and other digested fragments in the reaction mixture (Fig. 2A). The retention volume (RV) of F(ab')₂ and Fc were 49.00 and 53.88 mL, respectively. From the UV profile of the RM, we can see that the distance between these two peaks were away from each other enough for a successful separation. There were also small amounts of other digested fragments with low molecular weight (RV = 58.11 and 64.80 mL) that appeared in the FPLC UV profile of the RM, which were far away from the F(ab')₂ peak and did not impact the purification. The F(ab')₂ peak was collected and re-injected into the FPLC for quality control (QC; Fig. 2B). The QC profile clearly indicated that the F(ab')₂ fragment was pure (single peak) and there were no other protein contaminations in the collection.

For further characterization, the full antibody, pepsin digested reaction mixture, and the FPLC-purified F(ab')₂ and Fc peaks, were loaded onto the same gel and run with nonreducing sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2C). On the gel, the reaction mixture lane clearly showed that there was no full antibody left (no band at 150 kD position), which indicated that the digesting reaction was completed. As expected, the bands of F(ab')₂ and Fc peaks were pure and located at the corresponding retention positions as of the 100 and 50 kD standard proteins, respectively. This result agrees well with the theoretic molecular weights of F(ab')₂ and Fc. Furthermore, the mass spectrometry (MALDI-TOF) characterization of the F(ab')₂ peak indicated that the collection was pure, and the accurate molecular weight of the collected F(ab')₂ fragment in this study was 97,218 Da (Supplementary Fig. S1A). There was a faint band beneath the F(ab')₂ band in Figure 2C, lane 3, this is a contamination or decomposed component in the original intact antibody stock. This faint band was removed during the following purification step for Df-F(ab')₂.

FIG. 1. Synthesis of ^{89}Zr -labeled antibody F(ab')₂ fragment. Color images are available online.

FIG. 2. Size exclusion FPLC UV profiles of the pepsin-digested antibody reaction mixture (A) and QC of the collected F(ab')₂ fragment (B). Peaks a and b represent the F(ab')₂ and the Fc fragments, respectively. Non-reducing SDS-PAGE characterization (C) of the SE FPLC-purified F(ab')₂ fragment. 1: standard protein ladder; 2: pepsin-digested antibody reaction mixture; 3: F(ab')₂ fragment collection from FPLC (peak a); 4: Fc fragment collection from FPLC (peak b). QC, quality control; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UV, ultraviolet. Color images are available online.

The bifunctional chelator Deferoxamine (Df) was successfully conjugated onto the molecule of F(ab')₂ by incubating the fragment with p-SCN-Bn-Deferoxamine at basic condition (pH 9.4) at RT for 2 h. The resulting Df-F(ab')₂ was purified with size exclusion columns, and characterized with mass spectrometry (MW: 98,025 Da; Supplementary Fig. S1B). The difference between the molecular weights of Df-(ab')₂ and F(ab')₂ indicated that there was a mixture of conjugates with various Df-to-F(ab')₂ ratios. Df-anti-PD-L1 was also successfully prepared with the full antibody and the p-SCN-Bn-Deferoxamine.

The murine melanoma cell line B16F10 has been shown to overexpress PD-L1 on its cell surface in the literature,^{26,27} which enables it for testing the PD-L1 binding affinity and specificity of various PD-L1-targeting ligands *in vitro* and *in vivo*. In this study, we first use flow cytometry to confirm the PD-L1 expression levels on the B16F10 cells cultured in our lab (Fig. 3A). Then, B16F10 cells were used for evaluation of PD-L1-targeting affinity of the F(ab')₂ and Df-F(ab')₂ *in vitro*. The result of the competitive binding assay (Fig. 3B) indicated that the IC₅₀ values for F(ab')₂ and Df-F(ab')₂ were 1.45 and

4.15 nM, respectively. Although the affinity of Df-F(ab')₂ was slightly lower than that of F(ab')₂ according to the IC₅₀ values, both proteins maintained the nano-molar binding affinity of the original antibody (0.32 nM) to the PD-L1 target.

To prepare ⁸⁹Zr-Df-anti-PD-L1 and ⁸⁹Zr-Df-F(ab')₂, Df-anti-PD-L1 or Df-F(ab')₂ was incubated with ⁸⁹Zr oxalate solution at pH 7.5 for 60 min at 37°C. Both ⁸⁹Zr-Df-anti-PD-L1 and ⁸⁹Zr-Df-F(ab')₂ showed greater than 98% radiochemical purities after NAP-10 column purification. The radiochemical yields were higher than 90%, and both ⁸⁹Zr-Df-anti-PD-L1 and ⁸⁹Zr-Df-F(ab')₂ possessed radioactive specific activity as high as 10 μCi/μg protein. The stability analysis with NAP-10 column showed that both ⁸⁹Zr-Df-anti-PD-L1 and ⁸⁹Zr-Df-F(ab')₂ were stable *in vitro* for up to a week at 4°C. About 96.9% and 88.9% of ⁸⁹Zr-Df-F(ab')₂ remained intact after incubation at 37°C for 2 and 24 h, respectively. When incubated with mouse serum, a majority of the ⁸⁹Zr-Df-F(ab')₂ remained intact as well (95.2% and 77.0% for 2 and 24 h, respectively).

To verify the PD-L1-targeting specificity of the antibody fragment post radiolabeling, freshly prepared ⁸⁹Zr-Df-F(ab')₂

FIG. 3. (A) Flow cytometry analysis of PD-L1 expression on B16F10 cells. Red: non-specific staining with APC-conjugated isotype IgG2b; Blue: staining with APC-conjugated anti-mouse PD-L1 antibody. (B) *In vitro* competitive binding assay of protein fragments. IC₅₀ values of F(ab')₂ and Df-F(ab')₂ were 1.45 and 4.15 nM, respectively. (C) *In vitro* B16F10 cell uptakes of ⁸⁹Zr-Df-F(ab')₂ with or without antibody (or its isotype IgG2b) blockage (1 μM). Student's *t*-test showed that cell bound ⁸⁹Zr-Df-F(ab')₂ radioactivity was significantly blocked by anti-PD-L1 antibody, but not by isotype IgG2b. IC₅₀, inhibitory concentration of 50%; PD, programmed death. Color images are available online.

was incubated with overnight-seeded B16F10 cells, and competed with unmodified full antibody or isotype IgG2b (Fig. 3C). ^{89}Zr -Df-F(ab')₂ was taken up by B16F10 cells, and these uptakes were specifically blocked by unmodified anti-PD-L1 antibody (10^{-6} M; 80% blockage), but not by the same concentration of isotype IgG2b. This *in vitro* cell binding data clearly indicated that ^{89}Zr -Df-F(ab')₂ maintained the specific PD-L1 binding of the original full anti-PD-L1 antibody. To further verify that the modification on the terminal amine at the lysine side chain on the antibody or its F(ab')₂ fragment does not eliminate their PD-L1 targeting specificity and affinity, FITC-tagged proteins (FITC-anti-PD-L1, FITC-F(ab')₂, and FITC-isotype IgG2b) were prepared and used for B16F10 cell staining. FITC was used here because it uses the same functional group (isothiocyanate) to form covalent bonding with the amine on the lysine side chain as the p-SCN-Bn-Deferoxamine. The flow cytometry results (Supplementary Fig. S2) agreed well with their radiolabeled counterparts (Fig. 3C), FITC-F(ab')₂ and FITC-anti-PD-L1 had similar and much higher staining on B16F10 cells than the FITC-isotype IgG2b.

One goal of this study is to investigate whether the F(ab')₂ fragment possessed more favorable pharmacokinetics for *in vivo* imaging of PD-L1 levels than the full antibody; hence wild-type C57BL/6 mice were used here to compare their *in vivo* pharmacokinetic differences. Both ^{89}Zr -Df-anti-PD-L1 and ^{89}Zr -Df-F(ab')₂ were injected into C57 mice intravenously, and at selected time points, groups of mice were euthanized, and organs of interest were collected, weighted, and counted. The resulting biodistribution data (Fig. 4; Supplementary Table S1) showed that the uptakes of ^{89}Zr -Df-anti-PD-L1 and ^{89}Zr -Df-F(ab')₂ in the brain, lungs, pancreas, stomach, intestines, muscle, and skin were similar, but significantly different in liver and spleen. Although not significant, the kidney uptake of ^{89}Zr -Df-F(ab')₂ was higher than that of ^{89}Zr -Df-anti-PD-L1 at 24 h post injection ($13.09 \pm 2.1\%$ ID/g vs. $7.30 \pm 1.91\%$ ID/g). The liver uptake of ^{89}Zr -Df-F(ab')₂

was 10.94 ± 1.29 and $8.43 \pm 0.31\%$ ID/g at 4 and 24 h, respectively. However, for the same time points, the liver uptake of ^{89}Zr -Df-anti-PD-L1 was much higher, with 55.67 ± 12.21 and $54.47 \pm 6.48\%$ ID/g, respectively. Spleen uptake of ^{89}Zr -Df-F(ab')₂ (31.37 ± 7.66 and $29.65 \pm 1.71\%$ ID/g for 4 and 24 h, respectively) was also significantly reduced, comparing with the uptake of ^{89}Zr -Df-anti-PD-L1 (51.77 ± 6.57 and $64.7 \pm 3.71\%$ ID/g, respectively). These biodistribution data indicated that ^{89}Zr -Df-anti-PD-L1 and ^{89}Zr -Df-F(ab')₂ possessed very different *in vivo* distribution patterns, with ^{89}Zr -Df-F(ab')₂ showing much lower normal organ background signals.

The *in vivo* PD-L1 targeting ability of ^{89}Zr -Df-F(ab')₂ was evaluated in a B16F10 flank mouse model. The PET/CT imaging of ^{89}Zr -Df-F(ab')₂ in B16F10 melanoma-bearing mice was successfully conducted, and compared with the corresponding ^{89}Zr -labeled full antibody (Fig. 5). The PD-L1 receptors inside the B16F10 flank tumor were clearly visualized as early as 2 h post dose injection, and such signals had good retention 48 h post dose injection for ^{89}Zr -Df-F(ab')₂. However, for the ^{89}Zr -Df-anti-PD-L1, the tumor uptakes were much lower at the same time points, and the majority of the radioactivity accumulated in the liver and spleen. These PET images clearly showed that ^{89}Zr -Df-F(ab')₂ possesses superior pharmacokinetics and imaging contrast over its radiolabeled full antibody counterpart, with earlier and much higher tumor uptake (5.5 times more at 2 h post injection) and much lower liver background (51% reduction at 2 h post injection). Both ^{89}Zr -Df-F(ab')₂ and ^{89}Zr -Df-anti-PD-L1 showed accumulation in the brown adipose tissue in the mice (Supplementary Fig. S3), which agrees well with the literature of radiolabeled anti-PD-L1 antibodies.^{12,14} Kikuchi and coworkers used the same ^{89}Zr -labeled antibody for assessing PD-L1 levels in a B16F10 melanoma mouse model.²⁸ Although it was hard to compare our current data (24 h post injection, nonirradiated, antibody fragment) with Dr. Kikuchi's biodistribution data (48 h post injection, irradiated, full antibody), the same trend with high

FIG. 4. Biodistribution of ^{89}Zr -Df-F(ab')₂ (2, 4, and 24 h post dose injection) and ^{89}Zr -Df-anti-PD-L1 (4 and 24 h post dose injection) in naïve C57 black mice. Color images are available online.

FIG. 5. CT (A, C) and PET/CT (B, D) imaging of ^{89}Zr -Df-F(ab')₂ (C, D) and ^{89}Zr -Df-anti-PD-L1 (A, B) in B16F10 melanoma-bearing C57 mice 24 h post dose injection. Organ uptakes (E) of ^{89}Zr -Df-F(ab')₂ and ^{89}Zr -Df-anti-PD-L1 in B16F10 melanoma-bearing C57 mice. Color images are available online.

uptake in the spleen and liver was observed in both studies. In Dr. Kikuchi's study, a specific activity of $2 \mu\text{Ci}/\mu\text{g}$ was achieved, and a dose of $50 \mu\text{g}$ protein ($100 \mu\text{Ci}$) was used for injection for PET/CT imaging. In our study, the specific activity was $10 \mu\text{Ci}/\mu\text{g}$, and we used $0.1 \mu\text{g}$ protein ($1 \mu\text{Ci}$) for the biodistribution study and $17.6 \mu\text{g}$ protein ($176 \mu\text{Ci}$) for the PET imaging study. Heskamp and his coworkers have also shown a dose-dependent uptake (a range of 1 – $1000 \mu\text{g}/\text{mouse}$ was tested) of ^{111}In -labeled anti-PD-L1 antibody in a subcutaneous Renca tumor model with a different anti-PD-L1 antibody.¹² Hence, differences in uptake levels between Dr. Kikuchi's data and ours could be due to differing antibody doses.

In the tumor microenvironment, besides the B16F10 cells, infiltrating immune cells also express PD-L1. To determine what contributes to the PET signals, the *ex vivo* tumor samples were collected right post the last PET/CT scan, and stained and analyzed with flow cytometry. As Figure 6 indicated, although the majority of the cells in the B16F10 tumor were CD45[−] cells (mostly B16F10 cells and some stroma cells), only a small portion of CD45⁺ cells (immune cells) inside the tumor, the average PD-L1 receptor density on these immune cells were much higher than that on the nonimmune cells. According to these flow cytometry

data, both B16F10 and infiltrating immune cells contributed to the PET signals of PD-L1 level in the melanoma mouse (Fig. 6, Supplementary Figs. S4 and S5). Kikuchi and coworkers used the same ^{89}Zr -labeled intact antibody to evaluate the PD-L1 upregulation in B16F10 tumors.²⁸ Their finding also indicated that the PD-L1 levels on CD45⁺ cells were higher than that on the CD45[−] cells inside the B16F10 tumor, which agreed well with our findings in this study,

FIG. 6. Percentage of CD45⁺ and CD45[−] cells (A) and their corresponding mean fluorescent intensity of PD-L1 (B) within the B16F10 subcutaneously inoculated flank tumor. Color images are available online.

although the percentage of CD45⁺ cells was much higher in Dr. Kikuchi's study compared with ours.

Conclusions

A radiolabeled anti-PD-L1 F(ab')₂ fragment ⁸⁹Zr-Df-F(ab')₂ was successfully synthesized and showed superior imaging characteristics compared with its full antibody counterpart in PET imaging. These promising data suggest its potential for *in vivo* PET imaging of murine PD-L1 levels in melanoma and other cancer mouse models.

Authors' Contributions

C.B.: conducted experiments including the antibody digestion, chelator conjugation, radiolabeling, *In vitro* cell assay, and *In vivo* biodistribution and imaging. A.G.: conducted experiments including flow cytometry analysis of cell and *ex vivo* samples. X.H.: assisted flow cytometry analysis. J.A.B.: assisted mass spectrometry analysis. H.-G.Z.: assisted FPLC analysis, J.Y.: designed and supervised the flow cytometry analysis. H.G.: corresponding author; designed and supervised the entire study; prepared the article.

Acknowledgment

We thank Huaiyu Zheng from the University of Louisville Radiology Small Animal Imaging Core for his technical assistance with the PET/CT scans.

Disclosure Statement

No competing financial interests exist.

Funding Information

This work was supported in part by the Jewish Heritage Fund for Excellence and UofL Research II award (H.G.). C.B. was supported by the NCI Cancer Education Program (R25-CA134283). H.-G.Z. was supported by a VA Research Career Scientist (RCS) Award.

Supplementary Material

Supplementary Figure S1
Supplementary Figure S2
Supplementary Figure S3
Supplementary Figure S4
Supplementary Figure S5
Supplementary Table S1

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