Concurrent Injection of Unlabeled Antibodies Allows Positron Emission Tomography Imaging of Programmed Cell Death Ligand 1 Expression in an Orthotopic Pancreatic Tumor Model

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ABSTRACT: Among the treatment options for pancreatic ductal adenocarcinoma (PDAC) are antibodies against the programmed cell death receptor 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway. Positron emission tomography (PET) has been successfully used to assess PD-1/PD-L1 signaling in subcutaneous tumor models, but orthotopic tumor models are increasingly being recognized as a better option to accurately recapitulate human disease. However, when PET radiotracers have high uptake in the liver and spleen, it can obscure signals from the adjacent pancreas, making visualization of the response in orthotopic pancreatic tumors technically challenging. In this study, we first investigated the impact of radioisotope chelators on the biodistribution of [64Cu]-labeled anti-PD-1 and anti-PD-L1 antibodies and compared the distribution profiles of anti-PD-1 and anti-PD-L1 antibodies. We then tested the hypothesis that co-injection of unlabeled antibodies reduces uptake of [64Cu]-labeled anti-PD-L1 antibodies in the spleen and thereby permits accurate delineation of orthotopic pancreatic tumors in mice. Procedures: We established subcutaneous and orthotopic mouse models of PDAC using KRAS* murine pancreatic cancer cells with a doxycycline-inducible mutation of KRAS. We then (1) compared the biodistribution of [64Cu]-labeled anti-PD-1 with 2-(4-isothiocyanatobenzyl)-1,4,7,10-tetraazacyclododecane tetraacetic acid (p-SCN-Bn-DOTA) and 2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) used as the chelators in the orthotopic model; (2) compared the biodistribution of [64Cu]-Cu-NOTA-anti-PD-1 and [64Cu]-Cu-NOTA-anti-PD-L1 in the orthotopic model; and (3) imaged subcutaneous and orthotopic KRAS* tumors with [64Cu]-Cu-NOTA-anti-PD-L1 with and without co-injection of unlabeled anti-PD-L1 as the blocking agent. Results: [64Cu]-Cu-NOTA-anti-PD-L1 was a promising imaging probe. By co-injection of an excess of unlabeled anti-PD-L1, background signals of [64Cu]-Cu-NOTA-anti-PD-L1 from the spleen were significantly reduced, leading to a clear delineation of orthotopic pancreatic tumors. Conclusions: Co-injection with unlabeled anti-PD-L1 is a useful method for PET imaging of PD-L1 expression in orthotopic pancreatic cancer models.

INTRODUCTION

Pancreatic ductal adenocarcinoma (PDAC) is a lethal disease with a 5 year overall survival rate below 7%. Owing to the difficulty of early detection, many patients with PDAC are present with locally advanced or metastatic disease that cannot be surgically resected. These patients are commonly treated with conventional chemotherapy and/or radiotherapy, but outcomes remain abysmal despite vigorous research efforts.

Blockade of the programmed cell death receptor 1 (PD-1)/programmed cell death ligand 1 (PD-L1) pathway, by either anti-PD-1 antibodies or anti-PD-L1 antibodies, is a highly promising therapy that has elicited durable antitumor responses in a variety of tumor types, including melanoma and nonsmall cell lung cancer. However, antibodies against the PD-1/PD-L1 pathway have limited success in PDAC in the clinic, possibly due to the co-existence of multiple factors including an immunosuppressive tumor microenvironment, low tumor immunogenicity, and scarcity of tumor-infiltrating cytotoxic T cells. In preclinical studies, various efforts have been made to overcome resistance to anti-PD-1 or anti-PD-L1 antibodies by combining these agents with other treatment modalities such as vaccines, chemotherapy, radiation therapy, and local tumor ablation techniques. These studies establish that combination therapy can reverse resistance to immunotherapy in PDAC. The PD-1/PD-L1 pathway mediates self-tolerance and immune suppression. While PD-1 is absent on resting T cells, PD-1 is expressed on activated T cells and on exhausted T cells during chronic infection. PD-1-expressing, tumor-infiltrating CD8 T cells are often impaired in terms of antitumor functions. PD-L1 is endogenously expressed on tumor cells, tumor-associated antigen-presenting cells, and fibroblasts. Immunotherapy-induced cytokines, especially interferon-γ derived from cytotoxic T cells, can also increase...
PD-L1 expression in the tumor microenvironment, which leads to adaptive resistance of tumors to immune checkpoint blockade therapy. Therefore, molecular imaging of the PD-1/PD-L1 pathway not only can help identifying the proper patient populations for immunotherapies but also can provide a valuable tool to monitor tumor response to combination therapies with anti-PD-1/anti-PD-L1 antibodies.

Molecular nuclear imaging is a powerful tool to non-invasively and quantitatively assess the pharmacokinetics of antibodies and the expression of cell surface markers. [64Cu]Cu²⁺ cations have been conjugated to an ectodomain of PD-1 protein with high binding affinity to PD-L1 but much smaller size (14 KDa) than antibody (150 KDa) for imaging PD-L1 expression on tumor-infiltrating lymphocytes via positron emission tomography (PET). [89Zr] (t½ = 78.4 h)-labeled pembrolizumab, a U.S. Food and Drug Administration-approved antihuman PD-1 antibody, successfully imaged lymphocyte infiltration of salivary and lacrimal glands in humanized mouse models at 72−168 h after radiotracer injection. Although [89Zr]-labeled antibodies in general yield an excellent tumor-to-background ratio due to clearance of radiotracers from normal organs at delayed times (72–168 h post-injection), the long half-life of [89Zr] raises concern over increased radiation exposure and potential radiotoxicity. [125I]-labeled nivolumab, another U.S. Food and Drug Administration-approved antihuman PD-1 antibody, was used to image PD-1 expression via single-photon emission computed tomography in a colon cancer xenograft model. However, PD-1-targeted imaging does not directly visualize the primary tumor; rather, it visualizes the trafficking of PD-1-expressing immune cells. In contrast, PD-L1-targeted imaging visualizes tumor cells and other stromal cells. PD-L1-targeted imaging has been realized using radiolabeled whole antibodies and antibody fragments. In addition to the use of ectodomain of PD-1, attempts using peptides that are smaller than antibodies for imaging PD-L1 expression have also been reported. These radiotracers displayed lower liver uptake but higher kidney uptake than antibodies.

Most preclinical imaging studies to date for PD-1 and PD-L1 expression were performed in subcutaneous xenograft mouse models, in which the tumors were inoculated away from the organs of the reticuloendothelial system, including the liver and spleen. However, subcutaneous models are increasingly recognized as inferior to orthotopic models for recapitulating cancer biology because the composition of the tumor-associated microenvironment dramatically differs between subcutaneous and orthotopic xenografts. The use of orthotopic models of PDAC can be extremely challenging because of the proximity of orthotopic pancreatic tumors to both liver and spleen, which can specifically or nonspecifically entrap radiolabeled antibodies. In particular, the spleen was reported to be a significant site of uptake for radiolabeled anti-PD-L1 antibodies. In a recent clinical study, PET imaging using [89Zr]-labeled nivolumab (anti-PD-1) revealed substantial signals from the liver and spleen even at 7 days after injection of imaging tracers. Vento et al. reported a PET study with [89Zr]-labeled atezolizumab (anti-PD-L1) in a patient-derived xenograft model of renal cell carcinoma in OD/SCID mice. The tumors were clearly visualized because they were inoculated at a site away from the liver and spleen.

Recently, co-injection of unlabeled anti-PD-L1 antibodies was found to enhance the delivery of radiolabeled anti-PD-L1 antibodies to subcutaneously inoculated melanoma xenografts. In the current study, we first investigated the impact of radioisotope chelators on the biodistribution of [64Cu]-labeled antibodies and compared the distribution profiles of anti-PD-1 and anti-PD-L1. We then tested the hypothesis that co-injection of unlabeled antibodies reduces the uptake of [64Cu]-labeled anti-PD-L1 antibodies in the spleen and thereby permits enhanced delineation of orthotopic PDAC.

**RESULTS AND DISCUSSION**

**Radiolabeling and Characterization.** The radiochemical yields for all [64Cu]-labeled antibodies were 60−70% on the basis of instant thin-layer chromatography (ITLC) analysis. Radiolabeling of DOTA-anti-PD-1 (Figure 1a) yielded greater than 95% radiochemical purity after purification through a PD-10 column (Figure 1b). Similarly, radiolabeling of NOTA-anti-PD-1 and NOTA-anti-PD-L1 yielded greater than 95% radiochemical purity after PD-1 column purification (Supporting Information, Figure S1). The mean specific activity values for [64Cu]Cu-DOTA-anti-PD-1 and [64Cu]Cu-NOTA-anti-PD-1 (n = 3) and [64Cu]Cu-NOTA-anti-PD-1 (n = 8) in mice bearing orthotopic KRAS tumors at 24 h after injections is presented as mean ± standard error of the mean (SEM). The significance of differences was determined using the two-sided Student’s t-test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001, n.s., not significant.

![Figure 1](https://dx.doi.org/10.1021/acsomega.9b03731)
ant-PD-L1 bound to PD-L1 with essentially the same binding affinities, with $K_{D1}$ of $\sim 25$ nM and $K_{D2}$ of $\sim 624$ nM when sensorgram curves were fitted to a bivalent binding model (Supporting Information, Figure S2). On the basis of mass spectrometry data, each anti-PD-L1 contained an average of $\sim 3$ NOTA molecules (Supporting Information, Figure S3).
Impact of Chelators on Biodistribution of Anti-PD-1.

In mice bearing orthotopic KRAS* tumors, 24 h after antibody injection, most [64Cu]Cu-DOTA-anti-PD-1 was entrapped in the liver (29.6 ± 2.9% ID/g) and spleen (11.3 ± 2.6% ID/g) and the uptake of [64Cu]Cu-DOTA-anti-PD-1 in the tumor was only 0.3 ± 0.1% ID/g (Figure 1c). In contrast, at the same time point, significantly less [64Cu]Cu-NOTA-anti-PD-1 was entrapped in the liver (5.4 ± 0.3% ID/g) and spleen (6.3 ± 0.6% ID/g) than [64Cu]Cu-DOTA-anti-PD-1, and there was a significantly greater uptake of [64Cu]Cu-NOTA-anti-PD-1 than that of [64Cu]Cu-DOTA-anti-PD-1 in the tumor (5.5 ± 0.2% ID/g), blood (9.3 ± 0.7% ID/g), and lymph node (8.1 ± 0.8% ID/g) (Figure 1c). These data suggested that considerable trans-chelation of 64Cu2+ occurred with [64Cu]Cu-DOTA-labeled antibodies in the liver and spleen, in agreement with findings by other researchers. Therefore, NOTA was used for antibody radiolabeling in further studies.

It is interesting to note that the radioactivity in the intestine did not increase in mice injected with [64Cu]Cu-DOTA-anti-PD-1 compared to that in mice injection with [64Cu]Cu-NOTA-anti-PD-1, as is usually the case for a hepatobiliary excretion of the copper compounds after a trans-chelation. It is possible that liver metabolism and the excretion rate of [64Cu]Cu-DOTA-anti-PD-1 were delayed. Further studies including biodistribution studies at different time points after radiotracer injection are needed to see whether hepatobiliary excretion would occur at later time points.

Biodistribution of [64Cu]Cu-NOTA-anti-PD-1 and [64Cu]Cu-NOTA-anti-PD-L1. We then quantified the biodistribution of [64Cu]Cu-NOTA-anti-PD-1 and [64Cu]Cu-NOTA-anti-PD-L1 along with their respective isotype IgG controls in mice bearing orthotopic KRAS* tumors. Twenty four hours after radiotracer injection, the distribution pattern of anti-PD-1 was indistinguishable from that of its IgG control (Figure 2a). In contrast, at the same time point, most anti-PD-L1 had disappeared from the blood circulation and redistributed into other organs (Figure 2b). The uptake of anti-PD-L1 in blood was less than 5% that of IgG control, while the uptake of anti-PD-L1 in the liver, spleen, lung, intestine, and mesenteric lymph nodes was significantly higher than that of IgG control (p < 0.05). There was no significant difference in the tumor uptake of anti-PD-L1 and its IgG control (p > 0.05). However, the tumor-to-blood ratio of anti-PD-L1 was over 40 times higher than the tumor-to-blood ratio of anti-PD-1 or the two IgG controls (Figure 2c). These results indicated that [64Cu]Cu-NOTA-anti-PD-L1 is suitable for PET imaging of PD-L1 expression in mice.

Figure 4. Effect of the blocking ratio on microPET/CT imaging of orthotopic KRAS* tumors with [64Cu]Cu-NOTA-anti-PD-L1. [64Cu]Cu-NOTA-anti-PD-L1 was intravenously injected alone or with unlabeled anti-PD-L1 24 h prior to PET imaging. (a) Representative axial and coronal maximum intensity projection of microPET/CT images of [64Cu]Cu-NOTA-anti-PD-L1 alone or with a 15- and 50-fold excess of unlabeled anti-PD-L1. The spleen is indicated by the white solid arrow. Tumors are circled by yellow dashed circles. (b) Biodistribution of [64Cu]Cu-NOTA-anti-PD-L1 by the ex vivo cut-and-count method. BAT, brown adipose tissue. Three–four mice were included in each group. Data are presented as mean ± SEM. The significance of differences was determined using one-way ANOVA (n = 4/group). *p < 0.05, **p < 0.01, ***p < 0.001, n.s. not significant.
MicroPET/CT Imaging of Subcutaneous Tumors with [⁶⁴Cu]Cu-NOTA-anti-PD-L1. We then performed a proof-of-concept imaging study in the subcutaneous KRAS* model. As shown in Figure 3a, left panel, microPET/CT with [⁶⁴Cu]Cu-NOTA-anti-PD-L1 alone showed strong signals from the tumor, liver, spleen, lymph nodes, and brown adipose tissue. Others have also reported uptake of PD-L1-targeting radio-tracers in the brown adipose tissue. When 17-fold excess of unlabeled anti-PD-L1 was co-injected, however, there was a dramatic decrease in the signal intensity in the spleen, lymph node, and brown adipose tissue (Figure 3a, right panel). Consistent with the image-based analysis, the ex vivo biodistribution study performed immediately after imaging showed that blocking with unlabeled antibodies increased uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 by 13 times in blood and reduced its uptake in the spleen by 60% (p < 0.0001) (Figure 3b). There was no significant change in liver uptake of the radiotracer with or without blocking. Blocking doubled the uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in the tumor (p < 0.001). Immunohistochemical staining showed a high expression of PD-L1 in the spleen and tumor and moderate expression in the liver (Figure 3c). These data suggested that co-injection of unlabeled antibodies prevented entrapment of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in the spleen and prolonged its blood retention, thereby leading to the accumulation of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in the KRAS* tumors.

MicroPET/CT Imaging of Orthotopic Tumors with [⁶⁴Cu]Cu-NOTA-anti-PD-L1. We then performed microPET/CT imaging of orthotopic KRAS* tumors with [⁶⁴Cu]Cu-NOTA-anti-PD-L1 (13 μg, 150 μCi) was intravenously injected alone (n = 3) or with 200 μg of unlabeled anti-PD-L1 24 h prior to PET imaging (n = 5). Representative microPET images of live mice and dead mice with dissected tumors and the spleen following injection of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 alone (left panel) or with unlabeled anti-PD-L1 (right panel), along with corresponding photographs of dead mice. Corresponding autoradiographs and H&E-stained 20 μm thick tumor sections. Consistent with the image-based analysis, the ex vivo biodistribution study performed immediately after imaging showed that blocking with unlabeled antibodies increased uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 by 68% (p < 0.05). There was a significantly lower tumor uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in mice co-injected with a 50-fold excess of unlabeled anti-PD-L1, suggesting that the tumor uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 was partially blocked at higher doses of the cold antibody.

We further verified these findings by postmortem imaging of the mice with orthotopic KRAS* tumors with dissected tumors and the spleen (Figure 5). When only [⁶⁴Cu]Cu-NOTA-anti-PD-L1 was injected, the spleen exhibited the strongest PET signals from the tumor and liver. However, when unlabeled anti-PD-L1 was co-injected, the uptake in the spleen was substantially reduced, allowing clear delineation of orthotopic KRAS* tumors in axial and coronal images (Figure 4a). The biodistribution study confirmed that mice co-injected with unlabeled anti-PD-L1 had significantly more [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in the blood and significantly less [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in the spleen, kidney, lung, and brown adipose tissue (Figure 4b). Compared to injection of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 alone, co-injection of a 15-fold excess of unlabeled antibodies increased the tumor uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 by 68% (p < 0.05). There was a significantly lower tumor uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 in mice co-injected with a 50-fold excess of unlabeled anti-PD-L1, suggesting that the tumor uptake of [⁶⁴Cu]Cu-NOTA-anti-PD-L1 was partially blocked at higher doses of the cold antibody.
signals, which completely masked the signals from orthotopic KRAS<sup>+</sup> tumors on live animal imaging (Figure 5a). In the presence of unlabeled antibodies, however, the multinodule KRAS<sup>+</sup> tumor was successfully delineated from the background. Postmortem imaging confirmed that the tumor nodules exhibited stronger signals than the spleen and liver did (Figure 5a). Autoradiography (Figure 5b) further confirmed increased tumor deposition of [64Cu]Cu-NOTA-anti-PD-L1 in mice co-injected with cold anti-PD-L1. Imaging-based quantification showed that with co-injection of unlabeled anti-PD-L1, the tissue uptake of [64Cu]Cu-NOTA-anti-PD-L1 in the tumor was 2.7 times what it was without blocking. And uptake of [64Cu]Cu-NOTA-anti-PD-L1 in the heart was 1.8 times what it was without blocking because blocking prolonged the blood retention of [64Cu]Cu-NOTA-anti-PD-L1. Co-injection of cold anti-PD-L1 decreased the uptake of [64Cu]Cu-NOTA-anti-PD-L1 in the spleen and BAT by about 60% (Figure 5c).

In this study, we first established that antibodies radiolabeled with 64Cu via the NOTA chelator had lower background signals in organs of the reticuloendothelial system and therefore are better imaging candidates than antibodies 64Cu-labeled via the DOTA chelator. We also found that whereas [64Cu]Cu-NOTA-anti-PD-1 had long circulating time in the blood, [64Cu]Cu-NOTA-anti-PD-L1 was cleared from the blood and effectively accumulated in the spleen and KRAS<sup>+</sup> tumors with a tumor-to-blood ratio above 15. Co-injection of unlabeled anti-PD-L1 significantly increased blood retention of [64Cu]Cu-NOTA-anti-PD-L1 and reduced its uptake in the spleen, thereby permitting satisfactory imaging of orthotopic KRAS<sup>+</sup> tumors.

Both DOTA and NOTA are widely used in radiometal labeling of antibodies. DOTA is a preferred chelator for 68Ga and 90Y labeling. However, [64Cu]Cu-DOTA-labeled antibodies have shown higher uptake in normal tissues, including the liver and spleen, than [64Cu]Cu-NOTA-labeled antibodies. Both [64Cu]Cu-DOTA and [64Cu]Cu-NOTA have similar kinetic inertness, although [64Cu]Cu-NOTA exhibits better in vivo stability than [64Cu]Cu-DOTA. Our findings are consistent with previous reports that the [64Cu]Cu-DOTA-antibody had 5 times more liver uptake than the [64Cu]Cu-NOTA-antibody. As a result, [64Cu]Cu-DOTA-anti-PD-1 had a low tumor uptake because only a small fraction of the injected tracer was available for tumor accumulation. Lower abundance of PD-1 than PD-L1 may also have contributed to a lower tumor uptake of [64Cu]Cu-NOTA-anti-PD-1 compared to [64Cu]Cu-NOTA-anti-PD-L1. Radiotracers with higher specific activity and injected at a lower dose than the dose used in the current study may improve tumor detectability with anti-PD-1.

The tumor-to-blood, tumor-to-liver, and tumor-to-spleen ratios at 24 h after [64Cu]Cu-NOTA-anti-PD-1 injection were only 0.86 ± 0.4, 1.47 ± 0.09, and 1.30 ± 0.08, respectively, and therefore rendered PET tumor imaging is impossible with this radiotracer. In contrast, blocking increased the tumor-to-liver ratio of [64Cu]Cu-NOTA-anti-PD-L1 from 2.5 ± 0.6 to 3.3 ± 0.2 and the tumor-to-spleen ratio from 0.8 ± 0.2 to 4.3 ± 0.4 in the subcutaneous KRAS<sup>+</sup> model (Supporting Information, Table S1). Moreover, blocking with a 15 times molar excess of cold anti-PD-L1 increased the tumor-to-liver ratio of [64Cu]Cu-NOTA-anti-PD-L1 from 1.4 ± 0.2 to 2.5 ± 0.3 and the tumor-to-spleen ratio from 0.5 ± 0.1 to 2.5 ± 0.1 in the orthotopic KRAS<sup>+</sup> model (Supporting Information, Table S2). Under such conditions, even though blocking reduced tumor-to-blood ratios from 19 ± 3 to 2.9 ± 0.2, the decreased tumor-to-blood ratio was more than compensated for by the increased tumor-to-liver and tumor-to-spleen ratios, resulting in significantly improved tumor detectability. Our data support the idea that the spleen acts as a “reservoir” or “sink” for anti-PD-L1. The positive staining of PD-L1 in the mouse spleen (Figure 3c) can be attributed to the presence of PD-L1-positive B cells, splenic dendritic cells, and macrophages. Blocking with unlabeled anti-PD-L1 increased the uptake of [64Cu]Cu-NOTA-anti-PD-L1 in the blood by 13 times and allowed accumulation of the radiolabeled antibodies in KRAS<sup>+</sup> tumors (Figure 3b). Our data suggest that the preferential uptake in the spleen decreased the availability of [64Cu]Cu-NOTA-anti-PD-L1 for uptake in the tumor, raising the possibility that blocking this sink effect with unlabeled antibodies makes the radiotracer available to tumors. Indeed, co-injection of 15-fold of unlabeled antibodies with [64Cu]Cu-NOTA-anti-PD-L1 allowed improved visualization of the orthotopic KRAS<sup>+</sup> tumors compared to images acquired without blocking (Figure 4). Increasing the ratio of cold anti-PD-L1 to [64Cu]Cu-NOTA-anti-PD-L1 by 50 did not further reduce background radioactivity but reduced the tumor uptake of the radiotracer compared to that observed at a 15:1 ratio. Thus, a molar ratio of 15−20 between cold anti-PD-L1 and [64Cu]Cu-NOTA-anti-PD-L1 appears to yield optimal imaging properties in mice. The total dose of anti-PD-L1 (≈10 mg/kg) aligned well with that used in human patients. Therefore, our results indicate that the same strategy may lead to satisfactory imaging of PDAC in patients.

**CONCLUSIONS**

Molecular imaging of the PD-1/PD-L1 pathway is critical to provide a personalized plan for immune checkpoint blockade therapy. We have demonstrated that the biodistribution of radiolabeled anti-PD-1 and anti-PD-L1 antibodies depends on the selection of radioisotope chelators, and we have established that radiolabeled anti-PD-L1 is more suitable than radiolabeled anti-PD-1 for PET imaging 24 h after injection. Co-injection of unlabeled anti-PD-L1 prevents entrapment of [64Cu]Cu-NOTA-anti-PD-L1 in the spleen and permits high-quality imaging of orthotopic pancreatic tumors. Our method provides a useful tool that can be readily translated into the clinic for imaging PD-L1 expression in PDAC and for treatment optimization and response monitoring.

**EXPERIMENTAL SECTION**

**Chemicals and Reagents.** Antimouse PD-L1 (clone 10F.9G2) and its isotype rat IgG2b (clone LTF-2) and antimouse PD-1 (CD279, clone J43) and its isotype polyclonal Armenian hamster IgG were obtained from Bio X Cell (West Lebanon, NH). Bifunctional chelators 2-(4-isothiocyanato benzyl)-1,4,7,10-tetraazacyclododecane tetaacetic acid (p-SCN-Bn-DOTA) and 2-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) were obtained from Macrocyclics (Plano, TX). PD-10 columns were purchased from GE Healthcare (Pittsburgh, PA). [64Cu]CuCl<sub>2</sub> (specific activity, 21.42 Ci/μmol) was obtained from the Cyclotron Radiochemistry Facility at The University of Texas MD Anderson Cancer Center (Houston, TX).

**Cell Line and Animal Models.** All animal studies were approved by the Institutional Animal Care and Use Committee.
and were carried out in accordance with institutional guidelines. KRAS\textsuperscript* murine pancreatic cancer cells with a doxycycline-inducible mutation of KRAS\textsuperscript{G12D} were cultured and maintained as described in previous publications.\textsuperscript{41} The orthotopic KRAS\textsuperscript* murine PDAC model was established by injecting KRAS\textsuperscript* cells into the pancreas head of 8-week-old female C57BL/6 mice (Taconic Biosciences, Rensselaer, NY). Briefly, the mouse was anesthetized with isoflurane (2% in O\textsubscript{2}), and a small incision was made at the left side of the abdomen to expose the spleen and pancreas. Then, 5 \times 10\textsuperscript{6} cells in 10 \muL of a one-to-one mixture of Hank’s balanced salt solution (HBSS) and Matrigel with reduced growth factors (Corning Inc., Corning, NY) were injected into the pancreas head using a 27-gauge needle. Ten seconds after the injection was completed, the needle was gently pulled out. The spleen and pancreas were placed back into the abdominal cavity, and the incision was closed with absorbable suture and clips. The subcutaneous KRAS\textsuperscript* model was established by subcutaneous injection of 1 \times 10\textsuperscript{6} cells in 100 \muL of HBSS at the right hind flank of C57BL/6 mice. The tumors were monitored by palpation and allowed to grow to about 6 mm in diameter before imaging studies.

Radiolabeling. Anti-PD-1 (1 nmol) was incubated with pSCN-Bn-DOTA (20 nmol) in phosphate-buffered saline (PBS) containing 50 \muM sodium bicarbonate (pH 8.5) at 4 \degree C for 4 h. Unreacted pSCN-Bn-DOTA was removed by passing the mixture through a PD-10 column using PBS as the eluent. The same procedures were used for the synthesis of NOTA-anti-PD-1, NOTA-anti-PD-L1, and NOTA-IgG controls. The PD-L1 binding affinity of purified NOTA-anti-PD-L1 was compared to that of the intact anti-PD-L1 using the surface plasmon resonance (SPR) technique. The number of NOTA molecules conjugated to each anti-PD-L1 in NOTA-anti-PD-L1 was characterized by liquid chromatography--mass spectrometry. Details for the SPR and mass spectrometry methods are provided in the Supporting Information.

For radiolabeling, each DOTA- or NOTA-conjugated antibody was incubated with \textsuperscript{64}Cu\textsuperscript{+} CuCl\textsubscript{2} in 0.1 M sodium acetate (pH 5) at 38 \degree C for 45 min. Free \textsuperscript{64}Cu\textsuperscript{+} cations were removed by passing the mixture through a PD-10 column eluted with PBS. Ethylenediaminetetraacetic acid (EDTA, 4 mM final concentration) was added to the reaction solution to scavenge excess \textsuperscript{64}Cu\textsuperscript{+} ions before PD-10 purification. The radiochemical purity of the resultant radiotracers was measured using instant thin-layer chromatography.

Biodistribution and Autoradiography. \textsuperscript{64}Cu-labeled antibodies were intravenously injected into KRAS\textsuperscript* bearing mice (3–8 mice per group) through the tail vein at a dose of 5–15 \mug per mouse, corresponding to the radioactivity of 1.1–3.7 MBq (30–100 \muCi) per mouse. Mice were euthanized 24 h after antibody injection. Radioactivity in the blood, tumor, and major organs was measured on a \gamma counter (Packard Cobra II, Perkin Elmer, Sugar Land, TX) and normalized to the weight of each organ. The whole-body distribution of antibodies was calculated as the percentage of injected dose per gram of the tissue (% ID/g) and expressed as mean \pm standard error of the mean (SEM).

For autoradiography, tumors were frozen in OCT blocks and cryosectioned into 10 \mum slices, which were then exposed to a Fuji image plate (BAS-SR 2025) for 24 h. The image plates were then scanned on a multifunctional imaging system (Fuji Film FLAS100, Life Science, Valhalla, NY).

MicroPET/CT Imaging. \textsuperscript{64}Cu-labeled anti-PD-L1 was dispersed in PBS and intravenously injected into KRAS\textsuperscript* bearing mice (3–5 mice per group) through the tail vein, at a dose of 15 \mu g/mouse, corresponding to the radioactivity of 5.5 MBq (150 \muCi) per mouse. For blocking studies, 250 or 750 \mug of unlabeled anti-PD-L1 was co-injected with the \textsuperscript{64}Cu-labeled anti-PD-L1. Twenty four hours after antibody injection, the mice were anesthetized with isoflurane (2% in O\textsubscript{2}) and placed in a Bruker Albitra PET/SPECT/CT scanner (Billerica, MA) in a prone position. The system had a field of view of 80 mm and a resolution of up to 1.3 mm for PET and 90 \mu m for CT along all three axes. MicroPET/CT images were acquired over a period of 15 min. For data analysis, the regions-of-interest (ROIs) were manually drawn to record the mean radioactivity and convert the values to units in KBq/cc. The results were then converted to % ID/cc. The microPET images were reconstructed using the three-dimensional maximum likelihood and ordered-subset expectation maximization algorithms. The CT images were obtained using a three-dimension-based, filtered back-projection algorithm. The fusion of PET and CT images was performed using PMOD base Functionality software version 4.0 (PMOD Technologies LLC, Zurich, Switzerland).

Statistical Analysis. Data were presented as mean \pm SEM. The significance of differences was determined with two-tailed Student’s t-test or one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test, using GraphPad Prism software 7 (La Jolla, CA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.9b03731.

Methods for surface plasmon resonance (SPR) binding assay and liquid chromatography--mass spectrometry; iTLC curves of \textsuperscript{64}Cu\textsuperscript{+}Cu-NOTA-anti-PD-L1, SPR sensorgrams of binding of anti-PD-L1 antibodies to immobilized PD-L1; mass spectra of NOTA-conjugated anti-PD-L1; tables summarizing tumor-to-organ ratios for biodistribution of \textsuperscript{64}Cu\textsuperscript{+}Cu-NOTA-anti-PD-L1 in the subcutaneous KRAS\textsuperscript* tumor model and in orthotopic KRAS\textsuperscript* tumor model (PDF)

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Notes
The authors declare no competing financial interest.

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