Cerenkov luminescence imaging is an effective preclinical tool for assessing colorectal cancer PD-L1 levels in vivo

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Abstract
Background: Preclinical and clinical studies have demonstrated that immunotherapy has effectively delayed tumor progression, and the clinical outcomes of anti-PD-1/PD-L1 therapy were related to PD-L1 expression level in the tumors. A 131 I-labeled anti-PD-L1 monoclonal antibody tracer, 131 I-PD-L1-Mab, was developed to study the target ability of non-invasive Cerenkov luminescence imaging in colorectal cancer xenograft mice.

Method: Anti-PD-L1 monoclonal antibody labeled with 131 I(131 I-PD-L1-Mab), and in vitro binding assays were used to evaluate the affinity of 131 I-PD-L1-Mab to PD-L1 and their binding level to different colorectal cancer cells, and compared with flow cytometry, western blot analysis, and immunofluorescence staining. The clinical application value of 131 I-PD-L1-Mab was evaluated through biodistribution and Cerenkov luminescence imaging, and different tumor-bearing models expressing PD-L1 were evaluated.

Results: 131 I-PD-L1-Mab showed high affinity to PD-L1, and the equilibrium dissociation constant was 1.069 × 10^(-9) M. The competitive inhibition assay further confirmed the specific binding ability of 131 I-PD-L1-Mab. In four different tumor-bearing models with different PD-L1 expression, the biodistribution and Cerenkov luminescence imaging showed that the RKO tumors demonstrated the highest uptake of the tracer 131 I-PD-L1-Mab, with a maximum uptake of 1.613 ± 0.738% ID/g at 120 h.

Conclusions: There is a great potential for 131 I-PD-L1-Mab noninvasive Cerenkov luminescence imaging to assess the status of tumor PD-L1 expression and select patients for anti-PD-L1 targeted therapy.

Background
Anti-tumor immunity is a dynamic process of constant rebalancing, the anti-tumor immunity inhibits the tumor growth, while the tumor evades anti-tumor immunity by modifying the surrounding tumor microenvironment (TME) [1]. Antigen-presenting cells (APCs) activate T cells through two signals to eliminate heterologous antigens in the body. The first signal is between the antigen-loaded major histocompatibility complex (MHC) molecules and the T cell receptor (TCR) to identify the antigens. B7-1/B7-2 protein that is expressed on the surface of APCs bind to the co-stimulatory
molecule CD28, further activating the T cells to form the secondary signal. To limit the possibility of excessive immune response that lead to tissue damage, T cells produce inhibitory molecules as a negative feedback mechanism, including the programmed cell death-1 (PD-1)[2]. The PD-1 protein has two ligands, programmed cell death ligand-1 (PD-L1) and programmed cell death ligand-2, in which PD-L1 expresses on the surface of tumor cells and bind to PD-1 expressing T cells, causing T cell exhaustion and evasion of immune surveillance. PD-L1 also binds to CD80, which competitively inhibits CD80-ligand-bound T-cell activation pathways[3].

Colorectal cancer (CRC) is one of the most common malignant neoplasms throughout the world, and its incidence and mortality ranked third among all the malignant neoplasms[4]. By 2030, its burden is expected to increase by 60%, and new cases were predicted to increase by 2.2 million, with 1.1 million cancer-related deaths[5]. Unfortunately, up-regulated PD-L1 expression can lead to poor prognosis in CRC patients[6], but the immune checkpoint inhibitors (ICIs) therapy has opened a new window, bringing hope to patients with high PD-L1 expression. Current clinical studies revealed that patients with MSI/dMMR mCRC and MSI/dMMR non-CRC chemo-resistance metastasis demonstrated better efficacy with anti-PD-L1 therapy, with objective response rates of 40% and 57%, respectively[7]. More interestingly, PD-L1 is expressed in a variety of cancers, and preclinical studies on the use of immunotherapy for many malignant tumors have achieved better results. Due to exciting efficacy results in patients with advanced or unresectable melanoma, Pembrolizumab has become the first FDA-approved ICI based on PD-1/PD-L1 signaling pathway[8]. Till date, anti-PD-1/PD-L1 therapy has been confirmed as an effective strategy and approved for treating a vast number of malignant neoplasms, including triple negative breast cancer[9], small cell lung cancer[10], Hodgkin lymphoma[11] and cervical cancer[12]. The anti-PD-1/PD-L1 therapies demonstrate better response in patients, and this is related to tumor PD-L1 expression level in vivo[13]. For response stratification and ideal patient selection, it is necessary to detect the tumor PD-L1 expression level. It is worth noting that the tumor cells are surrounded by a homeostatic and dynamic TME that involves many uncertain factors including cytokines secretion, hypoxia, inflammation and treatment responses, and changes in one of these factors affects tumor PD-L1 expression[14]. Hence, it is suspicious to
determine PD-L1 expression on tissue samples by immunohistochemical (IHC) analysis, and a more effective PD-L1 detection method is urgently required. Molecular imaging with radiolabeled anti-PD-L1 antibodies comprehensively and dynamically assesses tumor PD-L1 expression in vivo, and monitors the possible changes in tumor PD-L1 expression during treatment.

Our prior study[15] used near-infrared dyes to optically label anti-PD-L1 monoclonal antibodies, confirming the feasibility of non-invasive monitoring of PD-L1 expression in vivo. Therefore, this study aimed to develop a $^{131}$I-labeled anti-PD-L1 monoclonal antibody to determine the possibility of non-invasive imaging to evaluate PD-L1 expression levels of CRC in vivo. In the future, imaging information obtained through this technology assists in selecting potential beneficiaries and predicting treatment responses for anti-PD-L1 therapy in patients with CRC.

Methods

Cell Culture

All human CRC cells were kindly provided by Stem Cell Bank, Chinese Academy of Sciences (Shanghai, China) and maintained in a humidified incubator at 37°C in 5% CO$_2$. SW620 was cultured in RPMI 1640 medium (BI, CT, USA) supplemented with 10% fetal bovine serum (FBS; BI, CT, USA) and 1% penicillin-– Streptomycin (P/S). LS74T and LoVo cells were cultured in DMEM medium (Gibco, NY, USA) with 10% FBS and 1% P/S. RKO cells were maintained in MEM medium (Gibco, NY, USA) supplemented with 10% FBS, 1 mmol/L sodium pyruvate (Gibco, NY, USA) and 1% P/S.

Western Blot analysis

LoVo, SW620, LS174T and RKO were lysed in RIPA buffer with 1 mmol/L PMSF on ice for 30 min. Equal amounts of total cellular protein (30 μg) were dissolved in SDS-PAGE gel and then transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany). The PVDF membranes were blocked with PBST containing 5% BSA for 1 h followed by incubation overnight at 4 °C with anti-PD-L1 antibody (#ab205921, Abcam, Tkoyo, Japan) at 1/200 dilution and anti-β-actin (Proteintech, Wuhan, China) at 1/5000 dilution. After washing with PBST, the membranes were incubated with goat anti-rabbit horseradish peroxidase (HRP)-conjugated secondary antibody (ZSGB-BO, Beijing, China) for 1h at room temperature(RT). The bands were then detected by ChemiScope 6000 Exp.
chemiluminescence imaging system (Clinx, Shanghai, China) and β-actin quantitative normalization was performed using ImageJ 1.60 (NIH).

**Flow cytometry**

Briefly, the cells were washed thrice with staining buffer (PBS containing 2 mmol/L EDTA and 0.5% FBS), and then the cells were incubated with recombinant anti-PD-L1 antibody ( #ab205921, Abcam, Tkoyo, Japan) or control IgG ( #ab17273, Abcam, Tkoyo, Japan) at 1/100 dilution for 30 min at RT after blocking with goat serum for 1h. Next, the cells were washed and resuspended, stained with Alexa Fluor® 647 conjugated Donkey Anti-Rabbit IgG secondary antibody (Biolegend, CA, USA) at 1/1000 dilution for 30 min at RT without light, and analyzed on a Cyto-Flex flow cytometer (Beckman, CA, USA). At least 20,000 events were recorded and analyzed using FlowJo software VX0.7 (BD, NJ, USA).

**Immunofluorescence staining**

The cells at a density of 5×10⁵ per cell line were cultured in 6-well plates with sterile glass slides until the cells were grown on the glass slides. The slides were then washed thrice with PBS and fixed in 4% paraformaldehyde for 15 min. Non-specific binding of antibodies was blocked by 10% goat serum for 30 min. The cells were incubated with anti-PD-L1 monoclonal antibody ( #ab205921, Abcam, Tkoyo, Japan) for overnight at 4 °C. A secondary Goat Anti-Rabbit antibody (Auragene, Changsha, China) conjugated with Dylight-488 was applied for 1 h at RT in a wet box. The nuclei were counterstained with DAPI medium. The images were obtained using an inverted fluorescence microscope (Nikon, Tokyo, Japan).

**Radiolabeling of antibody**

For ¹³¹I radiolabeling, anti-PD-L1 monoclonal antibodies were used with Na¹³¹I (Chengdu Gaotong Isotope Co, China) and 1,3,4,6-Tetrachloro-3α,6α-diphenylglycouril ( Iodogen®, Sigma, MO, USA) as described previously[16]. Briefly, in a tube coated with 50 μg Iodogen, 100μl 6.91nmol/L anti-PD-L1 monoclonal antibody ( #HY-P9904, MCE, NJ, USA) 0.25 mol/L phosphate buffer (pH 7.6), and 18.5 MBq of Na¹³¹I were added into the tube. All components of the tube were blended frequently, and the reaction was carried out at RT for 15 min. Subsequently, the contents were transferred onto a PD-10
column (GE Healthcare, MA, USA) and purified by using 20 mmol/L PBS containing 0.5% BSA as eluent (pH 7.4). 1 ml fraction of the eluate was collected and the radioactivity in 1μl aliquots of each fraction was measured by using a PerkinElmer 2480 automatic γ-counter, wherein the radiochemical purity was >95%.

**In vitro assay**

Human CRC cell lines LoVo, SW620, LS174T and RKO were adjusted to a concentration of 5×10⁶/ml. RPMI-1640 containing 0.5% BSA was used as the binding buffer, followed by incubation of 100μl fractions of cell suspension with 100μl 47.5pmol/L ¹³¹I-PD-L1-Mab (1.3kBq) at 37°C for 1 h as the test group (X group). The residual group (O group) and the total dose group (T group) for the controls were added with the same amount of ¹³¹I-PD-L1-Mab, and binding buffer was added to make the liquid volume equal. To determine the non-specific binding (NSB), ¹³¹I-PD-L1-Mab was incubated with RKO cells in the presence of a 2000-fold excess of unlabeled PD-L1 antibody. After cell incubation, the X group, O group and NSB group were separated by centrifugation (1300g, 10min) to obtain the protein-binding fraction (pellet) and then the supernatants were removed. After centrifugation, the counts per minute (cpm) were measured in each group with a γ-counter (PerkinElmer). These were conducted in triplicate, and the cell-binding ratio was calculated using the formula,

\[
\frac{X_{cpm}(NSB_{cpm}) - O_{cpm}}{T_{cpm}} \times 100\%
\]

In a competitive inhibition assay, ¹³¹I-PD-L1-Mab (750Bq) was incubated with RKO cells in the presence of varying concentrations of the unlabeled antibody (11.5-2300pmol/L) at 37°C for 1 h within 1ml of binding buffer. After incubation, the cell components were obtained by centrifugation, and the cell-associated activities were measured in a shielded well-type γ-counter. The IC50 value is defined as the concentration of unlabeled antibody that is required for 50% inhibition of radiolabeled antibody. In saturation binding assay, increasing concentrations of ¹³¹I-PD-L1-Mab (65-3333Bq) were incubated with 5 ×10⁵ RKO cells in 1 ml binding buffer at 37°C for 1 h. To detect non-specific binding,
a 200 molar ratio of unlabeled antibody was used for co-incubation. Specific binding is defined as the binding of PD-L1 antibody and PD-1 antigen expressed on tumor cells membrane, which is equivalent to the difference between total binding and non-specific binding. The GraphPad Prism 7.00 software fits the curve on the relationship between specific binding and non-specific binding to determine PD-L1 receptor density of each cell and the dissociation constant (Kd) of the $^{131}$I-PD-L1-Mab.

**Animal model**

Female BALB/C nu'/nu' mice (6-8 weeks old) were obtained from Changzhou cavens Laboratory Animal Co., Ltd. All animal protocols have been approved by the Animal Ethics Committee Board of Second Affiliated Hospital of Harbin Medical University (KY2018-215), and all procedures are under the National Institutes of Health guide for the care and use of Laboratory animals. Mice were housed in sterile cages with specific pathogen-free (SPF)-class animal facility on a 12 h light/dark cycle at 18 °C - 23 °C in 50% - 60% relative humidity. The mice had free access to food and drinking water, and were transfected with 200μl 2.5×10^7/ml human CRC cells subcutaneously in their right flank. Tumors were grown for more than 21 days until the average tumor volume reaches to approximately 500 mm³.

**Ex vivo biodistribution**

To determine the biodistribution and specificity of binding of $^{131}$I-PD-L1-Mab, two sets of study were performed in subcutaneous CRC cell line xenograft mice. Forty eight hours before each study injection of $^{131}$I-PD-L1-Mab, 0.5% sodium iodide solution was used instead of drinking water to prevent the enrichment of $^{131}$I in mouse thyroids.

In the first study, subcutaneous LoVo, SW620 and LS174T xenograft mice were divided into three groups to receive intravenous injections of 7.5kBq $^{131}$I-PD-L1-Mab, respectively. At 24 h, 48 h and 120 h after $^{131}$I-PD-L1-Mab injection, the mice were euthanized, and 100 μl of blood was drawn from the carotid artery. The major tissues, organs and tumor tissues of the mice were dissected and weighed, including the brain, heart, liver, spleen, lung, kidney, stomach, small intestine, colon, pancreas,
muscle, adipose, bladder and bone. All are placed in a γ-counting tube, and their radioactivity was measured using a γ-counter.

In the second study, subcutaneous RKO xenograft mice were divided into two groups. The control group was injected with 7.5kBq $^{131}$I-PD-L1-Mab, in the blocking group the subcutaneous RKO xenograft mice were injected with an excess of 300 μg of unlabeled PD-L1 antibody to block PD-L1 in vivo. The mice were euthanized at 48 h and 120 h after injection of $^{131}$I-PD-L1-Mab, and further processing was carried out according to the above steps.

**Cerenkov luminescence imaging (CLI)**

The mice that inoculated with subcutaneous xenografts of LoVo, SW620, LS174T and RKO cells received intravenous injections of 37MBq $^{131}$I-PD-L1-Mab (protein dose 29.26 μg). At 24 h, 48 h, and 120 h after injection, the mice were anesthetized with 2% isoflurane. After that, the mice were subjected to CLI using the IVIS Spectrum Imaging System (PerkinElmer, MA, USA), and the parameters were set to Binning Factor 8, FOV 13.4cm, Exposure Time 300s. The mice were placed in supine position, with the tumors facing the lens during scanning, and were continuously anesthetized. The images obtained from scanning were passed through the Living Image® 4.5 Software to determine the fluorescence intensity of tumors and background.

**Statistical analysis**

All data were expressed as means±SD. The difference in the uptake of $^{131}$I-PD-L1-Mab was assessed using ANOVA with Student–Newman–Keuls method multiple comparison test. The receiver operating characteristic (ROC) curves were drawn to evaluate the diagnostic efficacy of tumor uptake at different time points on tumors PD-L1 expression, and the areas under the curves (AUCs) at different time points were compared using the $U$ tests. Statistical analysis was performed using GraphPad Prism version 7.00 and SPSS version 19.0 for Windows. A statistically significant difference was defined as $p$ value of <0.05.

**Results**

**Different CRC cell lines have various levels of PD-L1 expression**
To determine the expression of PD-L1 protein in four human CRC cell lines (LoVo, LS174T, SW620, and RKO) in vitro, western blotting, flow cytometry and immunofluorescence staining were conducted. The western blotting results showed (Figure 1) various levels of endogenous PD-L1 expression among the four cell lines, in which the RKO cells (0.591 ± 0.006) showed the highest expression, followed by LS174T (0.527 ± 0.005), SW620 (0.329 ± 0.006), and LoVo (0.153 ± 0.009), and the difference was statistically significant (p<0.001). To further detect the expression of PD-L1 on the plasma membrane among the four cell lines, the mean fluorescence intensity of the four cell lines (Figure 2) was measured by flow cytometry, and ranked as high to low: RKO, LS174T, SW620, and LoVo (595500 ± 2121.320, 372325.0 ± 374.059, 9533.0 ± 35.355, 2523.5 ± 67.175, respectively; p<0.001). Also immunofluorescence staining proved that PD-L1 protein was mainly located on the plasma membrane in the four cell lines, and a small amount of PD-L1 expression was observed in the cytoplasm (Figure 3). In these four CRC cell lines, the diversity of PD-L1 protein expression was confirmed by western blotting and flow cytometry results, and the graded expression was shown as RKO, LS174T, SW620 and LoVo, respectively.

**Specific binding characteristics of $^{131}$I-PD-L1-Mab and PD-L1 in vitro**

Firstly, $^{131}$I-labeled PD-L1 antibody was used for cell binding assay along with a constant number of cells (5×10^5) and a constant $^{131}$I-PD-L1-Mab concentration (47.5pmol/L). As shown in Figure 4A, the cell-binding ratio of $^{131}$I-PD-L1-Mab to RKO, SW620, LS174T and LoVo cells was 26.39%, 2.96%, 4.94% and 4.14%, respectively (p<0.001). Next, a 2000-fold excess of unlabeled PD-L1 antibody was added to $^{131}$I-PD-L1-Mab-incubated RKO cells, and the cell binding rate of $^{131}$I-PD-L1-Mab to RKO was decreased from 26.39% to 2.88% (Figure 4B).

Secondly, based on the characteristics of RKO to determine the high PD-L1 expression in vitro and the high binding rate in the binding assay, RKO cells were selected for conducting $^{131}$I-PD-L1-Mab saturation binding assay and competitive inhibition assay. In saturation binding assay, using the method invented by scatchard, the number of binding sites was quantitatively determined and was 113671 ± 4183 sites per cell, and the equilibrium dissociation constant of RKO was estimated to be
1.069 nmol/L. In competitive inhibition assay, the IC50 of the unlabeled PD-L1 antibody was 252.1 nmol/L. These data indicated that the $^{131}$I-PD-L1-Mab antibody specifically binds to tumor cells expressing PD-L1.

**Ex vivo biodistribution of $^{131}$I-PD-L1-Mab**

The biodistribution study was conducted using $^{131}$I-PD-L1-Mab in nude mice with SW620, LoVo, and LS174T xenograft tumors. At 24h, the uptake of $^{131}$I-PD-L1-Mab in SW620 tumors was $0.412 \pm 0.179\%$ ID/g, which was increased to a maximum of $0.690 \pm 0.299\%$ ID/g at 48h, but decreased to $0.411 \pm 0.210\%$ ID/g at 120h. In contrast, in LoVo and LS174T tumors, the uptake was decreased over time, with $0.602 \pm 0.322\%$ ID/g and $1.580 \pm 1.533\%$ ID/g at 24h, respectively (Figure 5). The normal main organs with high uptake rates included lung, liver, and spleen, and the uptake rates were gradually decreased with time. Among them, the lung had the highest uptake rate, and the nude mice with LS174T xenograft tumors as examples showed the highest uptake in lung ($12.747 \pm 1.429\%$ ID/g) among other normal main organs, followed by spleen ($6.292 \pm 2.023\%$ ID/g), liver ($5.784 \pm 1.079\%$ ID/g), and kidney ($4.636 \pm 0.877\%$ ID/g). At all three time points, $^{131}$I-PD-L1-Mab uptake was significantly higher in LS174T tumors than in SW620 and LoVo tumors. Interestingly, the tumor-blood ratio of SW620, LoVo and LS174T tumors was increased with time, reaching the highest value at 120h (Figure 5D). These data indicated that $^{131}$I-PD-L1-Mab discriminates between high and low expression of PD-L1 in CRC tumors. In the blocking biodistribution study of nude mice with RKO xenograft tumors, compared to tumor-blood ratio of the blocking group receiving excess unlabeled PD-L1 antibody, the tumor-blood of the control group was not significantly higher at 48h ($1.613 \pm 0.738\%$ ID/g), however, the tumor-blood ratio of the control group was increased significantly at 120h ($1.510 \pm 0.564\%$ ID/g). These results suggest specific binding of $^{131}$I-PD-L1-Mab to RKO tumors.

**Cerenkov PD-L1 specific imaging visualizes and distinguishes xenografts with low and high PD-L1 expression**

$^{131}$I-PD-L1-Mab PD-L1 specific imaging clearly visualized LoVo, LS174T, SW620 and RKO xenografts,
and the tumor-background ratio was increased significantly with increasing time (Figure 7), reaching maximum at 120h. Similarly, the implanted tumors better displayed at 120 h. The tumor-background ratio remained the highest at 120 hours after injection of $^{131}$I-PD-L1-Mab in RKO tumors (13.471 ± 3.571), followed by LS174T (11.630 ± 1.473), LoVo (7.403 ± 2.337) and SW620 (7.015 ± 1.975). The tumor-background ratio increases over time, and this trend was consistent with the tumor-to-blood ratio in the ex vivo biodistribution study. Cerenkov imaging showed high to moderate uptake of $^{131}$I-PD-L1-Mab in RKO and LS174T tumors, and low uptake in SW620 and LoVo tumors. This was confirmed in the ex vivo biodistribution study, and was consistent with the western blotting and flow cytometry results. These results demonstrated that RKO and LS174T tumors showed high PD-L1 expression, while SW620 tumors and LoVo tumors showed low PD-L1 expression. Based on the tumor-background ratio of CLI obtained at 24h, 48h and 120h, the ROC analysis showed that the tumor-background ratio can effectively discriminate the tumors with high and low PD-L1 expression. The AUCs were 0.861, 0.889, and 0.972, respectively, with no statistically significant difference in the AUCs at each time point (p> 0.05).

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3.1. Cerenkov PD-L1 specific imaging visualizes and distinguishes xenografts with low and high PD-L1 expression

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**Discussion**

The traditional IHC analysis has many limitations in evaluating the tumor PD-L1 expression in patients. Also, the IHC scoring-criteria are not uniform and different manufacturers produce PD-L1 antibodies with different scoring-criteria. There are four main clinical trial assays, including 28 – 8, 22C3, SP142 and SP263, in which the PD-L1 scores have lower consistency[17]. Secondly, during the process of tumor growth, metastasis and apoptosis, the TME remains unstable, and PD-L1 expression showed spatio-temporal dynamics, and furthermore showed intratumor and intertumor heterogeneity[18]. The IHC-based core needle biopsy only reflects PD-L1 expression level in a tiny fraction of a single tumor, but cannot comprehensively detect the situation of tumors throughout the body and the changes that might occur over time under conditions such as chemotherapy, radiotherapy and immunotherapy. Fianlly, another important factor that interferes with immunotherapy is the disordered blood supply vessels surrounding the tumors. Although IHC analysis showed that some cancer patients are suitable to use ICIs(such as PD-L1 antibodies), but effective drug concentration cannot be reached in their tumors internally, causing failed immunotherapy of tumors. In contrast, molecular imaging with radiolabeled anti-PD-L1 antibodies is considered to be more superior. Molecular imaging comprehensively detects PD-L1 expression in patients with tumors throughout the body, and analyzes the real-time dynamic analysis of possible changes in tumor PD-L1
expression during treatment. So far, many studies have used different radionuclides, such as ($^{99\text{m}}$Tc, $^{68}$Ga, $^{64}$Cu, $^{89}$Zr and $^{18}$F) to label PD-L1 monoclonal antibodies[19,20] or engineered PD-L1 specific peptides[21–23] in preclinical and clinical research. Some researchers have even found that atezolizumab labeled with $^{89}$Zr can predict the PFS and OS in patients better than the IHC-based core needle biopsy [20].

Recently, Cerenkov luminescence imaging is an emerging imaging strategy used to determine the location and margins of tumors during surgery[24], and has been used in radionuclide therapy monitoring[25], and external beam radiotherapy plan making[26]. Compared with other optical imaging methods, fluorescence of CLI comes from a well-known physical phenomenon in the decay process of nuclides. It does not require external excitation light to irradiate and avoid the reduction of the signal-to-noise ratio caused by reflection of excitation light. More importantly, $^{131}$I labeling method has passed the test of time and proved to be very simple and robust.

Since the PD-L1 antibody atezolizumab that is used for labeling is a full-molecule IgG, its molecular weight determines its biological half-life of several days. So $^{131}$I, a $\beta$- and $\gamma$-emitter with a long physical half-life ($T_{1/2} = 8.021$ d), has become our option for labeling. The advantage of $^{131}$I is that it can be used as a clinical imaging agent and internal irradiation treatment drug for thyroid diseases, and is widely used clinically and is relatively easy to obtain in the clinical and scientific research fields. To the best of our knowledge, our study is the first study to synthesize $^{131}$I-labeled PD-L1 monoclonal antibody, and successfully measure PD-L1 expression in human CRC subcutaneous xenograft mouse tumors with CLI[27]. By contrast, PET is a promising molecular imaging technique, it has a high spatial resolution and positioning capabilities, it can quantify the tumor uptake more accurately, but the disadvantage of this technique involves expensive equipment, long scanning time and tedious steps of antibody labeling. While CLI is cheap, simplicity to operate and has higher throughput.

To develop a new Cerenkov luminescent tracer to detect PD-L1 expression in CRC, four different CRC
cell binding assays were performed. The cell binding assay confirmed that RKO and LS174T demonstrated high to moderate expression levels of PD-L1, while SW620 and LoVo showed low levels of PD-L1 expression, and the cell-binding ratio was consistent with western blotting analysis and flow cytometry. In the presence of excess unlabeled PD-L1 antibodies, the binding of $^{131}$I-PD-L1-Mab to PD-L1 on to the surface of tumor cells was significantly blocked. This showed that $^{131}$I-labeled monoclonal PD-L1 antibody still retained its immunoreactivity and can specifically bind to PD-L1.

Secondly, a high affinity of $^{131}$I-PD-L1-Mab for PD-L1 was estimated by saturation binding assay (Kd = 1.069 nmol / L), and it is similar with that of the affinity of $^{111}$In-PD-L1 3.1 (Kd = 1.0 nmol / L) syntheses by Heskamp S, et al[28]. However, it might be due to the source of antibody or different labeled nuclides that change the immune characteristics of the antibody, and the values of Kd are not identical. The immunoreactivity of $^{131}$I-PD-L1-Mab and its high affinity to PD-L1 laid foundation to its good characteristics for in vivo imaging.

Biological distribution analysis showed that RKO xenografts have high uptake of $^{131}$I-PD-L1-Mab, LS174T xenografts showed moderate uptake, while the uptake of LoVo and SW480 was decreased significantly, and the tumor and spleen uptake of RKO tumor bearing mice were reduced by injecting excess unlabeled anti-PD-L1-Mab. This again verified that $^{131}$I-PD-L1-Mab specifically targets PD-L1 expression in tumor cells. Besides the tumor cells, PD-L1 also showed expression on hematopoietic cells[10] (such as B cells, T cells, megaphagocytes, and dendritic cells), in which the lung, spleen and liver are rich. On the other hand, atezolizumab has cross-reactivity between human and mouse[29], and it is possible to explain as to why a large amount of $^{131}$I-PD-L1-Mab was observed in lung, spleen and liver. Similar results were also observed in studies using a mouse cross-reactive PD-L1 antibody such as atezolizumab[28–30] or using rat anti-mouse antibodies in a mouse tumor model[31]. The uptake of $^{131}$I-labeled monoclonal PD-L1 antibody in four xenograft models with different levels of PD-L1 expression differed from each other in vivo, and the Cerenkov fluorescence intensity of PD-L1 high expression in tumors was significantly higher than that of PD-L1 low expression, which in turn was
affected by receiver operating characteristic curve analysis. The results also proved that PD-L1-specific CLI can effectively distinguish the level of PD-L1 expression in human CRC xenograft tumors. Some studies showed that radiotherapy with a certain dose effectively improved the response of anti-PD-L1 treatment in pancreatic cancer[32], cervical adenocarcinoma / adenosquamous carcinoma[33] and other cancers[34]. This might be due to the radiation-induced immunogenic cell death and the release of neoantigens in order to improve the recognition of CD8+ T cells and its killing effect on tumor cells, while promotion of the release of chemokines can attract effector T cells into the TME[14]. Imaging in the four groups of tumor bearing mice after few days revealed that the volume of some xenografts was slightly reduced (data not shown). Although the thymus of BALB/C immunodeficient mice was degenerated and could not generate immature B cells and T cells, the NK cells could still be developed and matured in the bone marrow. Irradiation of tumors with $^{131}$I upregulated MHC I or other antigen components presenting the mechanism, including the NK cell activating ligand, to improve the immunity of immunodeficient mice to xenografts. Similar results were observed in recent studies[27,35]. Correspondingly, in human CRC models, whether $^{131}$I-labeled anti-PD-L1 antibody has a potential synergistic effect over single immunotherapy needs further investigation.

Conclusion
In this study, a $^{131}$I-labeled anti-PD-L1 monoclonal antibody was synthesized to evaluate its properties both in vitro and in vivo.$^{131}$I-PD-L1-Mab accumulates in tumors in high to moderate levels of PD-L1 expression, and can be used in visualizing subcutaneously implanted xenografts more clearly. The CLI with $^{131}$I-PD-L1-Mab is not only considered as a prominent non-invasive imaging method that is accessible to assess and monitor dynamic PD-L1 expression in tumor lesions, but is also used to clinically screen potential patients who can benefit from PD-1/ PD-L1 targeted therapy. Meanwhile, further study regarding the synergistic therapeutic effects of $^{131}$I-PD-L1-Mab is necessary.

Abbreviations
TME: tumor microenvironment; APCs: Antigen-presenting cells; MHC: major histocompatibility complex; TCR: T cell receptor; PD-1: programmed cell death-1; PD-L1: programmed cell death ligand-
1; CRC: Colorectal cancer; ICIs: immune checkpoint inhibitors; IHC: immunohistochemical; NSB: nonspecific binding; CLI: Cerenkov luminescence imaging; ROC: receiver operating characteristic; AUCs: areas under the curves.

Declarations

Ethics approval and consent to participate

The study was approved by the Animal Ethics Committee Board of Second Affiliated Hospital of Harbin Medical University (KY2018-215).

Consent for publication

Not applicable.

Availability of data and material

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

HJJ, RJZ and ZHL contributed to the study concepts and to the study design. SZ, RJZ and WBP performed the experiments of this work. HJ and HBH contributed to the statistical analysis. HJJ and ZHL obtained the grant and supervised the project. SZ, HJJ, RJZ and WBP contributed to the manuscript preparation and to the manuscript editing and reviewing. All authors read and approved the final manuscript.

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Figures
Figure 1

PD-L1 total protein expression analysis of four CRC cell lines. A. Western blot analysis of total cell lysates using anti-PD-L1 and anti-\(\beta\)-actin antibody between LoVo, LS174T, SW620 and RKO in vitro. B. The ratio of PD-L1 total protein intensity. Data are expressed as means \(\pm\) SD, *** P <0.001, \((n = 3)\).
The differences in the PD-L1 expression on the plasma membrane among LoVo, LS174T, SW620 and RKO was evaluated by flow cytometry analysis. A. The analysis of anti-human PD-L1 antibody binding to the PD-L1 of plasma membranes in LoVo, LS174T, SW620 and RKO cell lines. B. Statistical summary of plasma membranes PD-L1 expression on four different CRC cell lines. Data are expressed as means ± SD, *** P < 0.001.
The subcellular localization of PD-L1 protein in LoVo, LS174T, SW620 and RKO was determined by immunofluorescence staining with anti-PD-L1 antibody (green) and DAPI nuclear staining (blue). PD-L1 protein is mainly located on the plasma membrane among the four cell lines, and a small amount of PD-L1 expression was observed in the cytoplasm.
Figure 4

Cell affinity characteristics of 131I-PD-L1-Mab in vitro. A. Cell binding of 131I-PD-L1-Mab to four different CRC cell lines with constant concentration of 131I-PD-L1-Mab. B. Cell binding of 131I-PD-L1-Mab to RKO with or without blocking. C. Saturation binding assay of 131I-PD-L1-Mab, Kd = 1.069nmol / L, the number of binding sites was 113671 ± 4183 per cell. D. Competitive inhibition assay of 131I-PD-L1-Mab showed an IC50 of 252.1 nmol/L. The data was expressed as means±SD, ***p<0.001.
Ex vivo biodistribution of 131I-PD-L1-Mab in SW620 (A), LoVo (B) and LS174T (C) tumor-bearing nude mice at 24 h, 48 h, and 120 h after injection. The mean (% ID/g) ± SD at each time point after injection (n = 4 mice at each time point). D. Corresponding quantitative data of the tumor (%ID/g) to blood (%ID/g) ratio of SW620, LoVo, and LS174T tumor-bearing nude mice.
Comparison of 131I-PD-L1-Mab tumor uptake and post-blocking uptake in subcutaneous RKO xenograft mice. A. After intravenous injection of 131I-PD-L1-Mab at 48h, blocking group mice was injected with an excess of unlabeled PD-L1 antibody. A. After intravenous injection of 131I-PD-L1-Mab at 120 h, the blocking group mice were injected with excess unlabeled PD-L1 antibody. The data was expressed as means (% ID/g) ± SD at each time point after injection, ***p<0.001.
PD-L1 specific CLI at different time points in human CRC xenograft models with 131I-labeled PD-L1 antibody. A. Cerenkov images of nude mice bearing four different CRC cell lines at 24 h, 48 h and 120 h. B. After injection of 131I-PD-L1-Mab (37MBq), the areas of interest were delineated along the tumor margin at 24 h, 48 h and 120 h to quantitatively measure the Cerenkov fluorescence intensity of the tumor to background ratio. C. The diagnostic effectiveness of Cerenkov images was tested by receiver operating characteristic curves. The data was expressed as means±SD, ***p<0.001.