The present invention relates to a method for detecting a PD-L1-positive tumor in a patient by imaging using a radiolabeled anti-PD-L1 antibody.
Non-invasive imaging of tumor PD-L1 expression

FIELD OF THE INVENTION

The present invention relates to a method for identifying patients suffering from or suspected to suffer from a cancer involving a solid tumor.

BACKGROUND OF THE INVENTION

T cells play an essential role in the anti-cancer immune response. T cell activation depends on the initial antigen-specific signal, presented via the antigen-loaded major histocompatibility complex (MHC) to the T cell receptor, and on activation of the costimulatory molecule CD28 by binding of CD80/86. T cells also express coinhibitory molecules that are capable of downregulating the immune response.(1) One major coinhibitory receptor is programmed death 1 (PD-1). PD-1 has two ligands, programmed death ligand-1 (PD-L1) and PD-L2, of which PD-L1 is most widely expressed. Binding of PD-L1 to PD-1 transduces an inhibitory signal to the T cell, resulting in inhibition of T cell proliferation, reduced secretion of effector cytokines, and potentially exhaustion. By up-regulating PD-L1 expression levels, tumor cells are capable of escaping immune recognition and attack.(2-4)

PD-L1 is expressed on a wide variety of tumors, including breast cancer, gastric cancer, renal cell cancer, ovarian cancer, non-small lung cancer, melanoma, and hematological cancers.(5) In general, PD-1 and PD-L1 have been demonstrated to be poor prognostic factors as high expression levels are associated with poor outcome of cancer patients.(5) Preclinical studies with anti-PD-1 and anti-PD-L1 antibodies have shown promising anti-tumor effects and have led to the initiation of several clinical investigations. Early clinical trials demonstrated objective and durable (>1 year) responses in patients with treatment-refractory, advanced melanoma, renal cell carcinoma, non-small cell lung cancer, and ovarian cancer.(6-11) Because of these impressive results, phase II/III studies are currently further exploring the therapeutic efficacy of these agents. Due to the impressive efficacy in melanoma patients, the FDA has recently granted accelerated approval of
pembrolizumab (anti-PD-1 antibody) for the treatment of patients with advanced or unreseetable melanoma following progression on prior therapies. (11)

Although studies with PD-1/PD-L1 targeted therapies show encouraging results, not all patients respond to this type of treatment and there is an urgent need for a predictive biomarker. In this regard, several studies have postulated that tumor PD-L1 expression might be required for response to anti-PD-1 and anti-PD-L1 targeted therapy. (7, 12-14) However, immunohistochemical analysis of PD-L1 expression as determined on archival tissue samples should be interpreted with caution, since PD-L1 expression may undergo changes due to alterations in tumor microenvironment or previous treatment. For example, interferon-γ (IFN-γ) and tumor necrosis factor-α (TNFa) can upregulate PD-L1 expression while vascular endothelial growth factor (VEGF) can downregulate tumor PD-L1 expression.(15-17) Also, certain chemotherapeutics such as doxorubicin can decrease the expression of PD-L1 on the tumor cell surface, while radiotherapy and paclitaxel are capable of upregulating PD-L1 expression.(18-20) There are no strict criteria to define PD-L1 positivity by immunohistochemistry (e.g. cytoplasmic versus membranous staining, number of positive tumor cells), and misinterpretation may occur due to heterogeneous expression within or between tumor lesions (e.g. primary versus metastatic lesions). (7, 12-14)

Therefore, there is still an unmet need for reliable methods for determining the level of PD-L1 expression in tumors.

SUMMARY OF THE INVENTION

The inventors have developed a non-invasive method for imaging PD-L1-positive tumors using radio-labelled anti-PD-L1 antibodies.

They have shown that it is possible to detect PD-L1-positive tumors and to discriminate between tumors having different expression levels of PD-L1.
Accordingly, the present invention relates to a labelled anti-PD-L1 antibody for use in a method of detecting a PD-L1-positive tumor in a patient, wherein said method comprises subjecting said patient to imaging.

In another aspect, the present invention relates to a method for detecting a PD-L1-positive tumor in a patient who has been administered a labelled anti-PD-L1 antibody, said method comprising subjecting the patient to imaging.

In another aspect, the invention relates to a method for monitoring the response to treatment in a patient suffering from cancer comprising the steps consisting of:

- carrying out the above method at a first time point t1;
- carrying out the above method at a second time point t2;

wherein a decrease in the expression level of PD-L1 observed at t2 compared to t1 is indicative of a response to treatment.

**DETAILED DESCRIPTION OF THE INVENTION**

As explained above, the inventors have developed an imaging technique based on the use of labelled anti-PD-L1 antibodies.

Said labelled anti-PD-L1 antibodies are targeted specifically to tumors expressing high levels of PD-L1 and can then be detected by suitable imaging techniques, which are known to the skilled person in the art.

Thus, the present invention relates to a labelled anti-PD-L1 antibody for use in a method of detecting a PD-L1-positive tumor in a patient, wherein said method comprises subjecting said patient to imaging.

PD-L1, also known as CD274, Programmed Cell Death 1 Ligand 1 (PDCD1LG1 or PD-L1) or B7-H1, is a type I transmembrane glycoprotein composed of IgC- and IgV-type extracellular domains, which binds to PD1.

The terms "PD1", "PD-1" and "Programmed cell death protein 1" refer to a member of the CD28 superfamily that delivers negative signals upon interaction with
its two ligands, PD-L1 or PD-L2. PD-1 and its ligands are broadly expressed and exert a wider range of immunoregulatory roles in T cells activation and tolerance compared with other CD28 members. PD-1 was isolated as a gene up-regulated in a T cell hybridoma undergoing apoptosis and was named program death 1.

As used herein, the term "antibody" has its general meaning in the art. The term "anti-PD-L1" antibody refers to an antibody that binds specifically to PD-L1. Preferably, said antibody does not bind to PD-L2.

Antibodies specifically directed against PD-L1 may be derived from a number of species including, but not limited to, rodent (mouse, rat, rabbit, guinea pig, hamster, and the like), porcine, bovine, equine or primate and the like. Antibodies from primate (monkey, baboon, chimpanze, etc.) origin have the highest degree of similarity to human sequences and are therefore expected to be less immunogenic. Antibodies derived from various species can be "humanized" by modifying the amino acid sequences of the antibodies while retaining their ability to bind the desired antigen. Antibodies may also be derived from transgenic animals, including mice, which have been genetically modified with the human immunoglobulin locus to express human antibodies. Procedures for raising "polyclonal antibodies" are well known in the art. For example, polyclonal antibodies can be obtained from serum of an animal immunized against PD-L1, which may be produced by genetic engineering for example according to standard methods well-known by one skilled in the art. Typically, such antibodies can be raised by administering PD-L1 protein subcutaneously to New Zealand white rabbits which have first been bled to obtain pre-immune serum. The antigens can be injected at a total volume of 100 µl per site at six different sites. Each injected material may contain adjuvants with or without pulverized acrylamide gel containing the protein or polypeptide after SDS-polyacrylamide gel electrophoresis. The rabbits are then bled two weeks after the first injection and periodically boosted with the same antigen three times at six weeks' interval. A sample of serum is then collected 10 days after each boost. Polyclonal antibodies are then recovered from the serum by affinity chromatography using the corresponding antigen to capture the antibody. This and
other procedures for raising polyclonal antibodies are disclosed by (Harlow et al, 1988), which is hereby incorporated in the references.

Although historically monoclonal antibodies were produced by immortalization of a clonally pure immunoglobulin secreting cell line, a monoclonally pure population of antibody molecules can also be prepared by the methods of the present invention.

Laboratory methods for preparing monoclonal antibodies are well known in the art (see, for example, Harlow et al, 1988).

A "monoclonal antibody" or "mAb" in its various names refers to a population of antibody molecules that contains only one species of antibody combining site capable of immunoreacting with a particular epitope. A monoclonal antibody thus typically displays a single binding affinity for any epitope with which it immunoreacts. Monoclonal antibody may also define an antibody molecule which has a plurality of antibody combining sites, each immunospecific for a different epitope. For example, a bispecific antibody would have two antigen binding sites, each recognizing a different interacting molecule, or a different epitope. As used herein, the terms "antibody fragment", "antibody portion", "antibody variant" and the like include any protein or polypeptide containing molecule that comprises at least a portion of an immunoglobulin molecule such as to permit specific interaction between said molecule and an antigen (e.g. PD-L1). The portion of an immunoglobulin molecule may include, but is not limited to, at least one complementarity determining region (CDR) of a heavy or light chain or a ligand binding portion thereof, a heavy chain or light chain variable region, a heavy chain or light chain constant region, a framework region, or any portion thereof, or at least one portion of a ligand or counter-receptor which can be incorporated into an antibody of the present invention to permit interaction with the antigen.

Monoclonal antibodies (mAbs) may be prepared by immunizing a mammal such as mouse, rat, primate and the like, with purified PD-L1 protein. The antibody-producing cells from the immunized mammal are isolated and fused with myeloma or heteromyeloma cells to produce hybrid cells (hybridoma). The hybridoma cells producing the monoclonal antibodies are utilized as a source of the desired monoclonal antibody. This standard method of hybridoma culture is described in (Kohler and Milstein, 1975). Alternatively, the immunoglobulin genes may be
isolated and used to prepare a library for screening for reactive specifically reactive antibodies. Many such techniques including recombinant phage and other expression libraries are known to one skilled in the art.

While mAbs can be produced by hybridoma culture the invention is not to be so limited. Also contemplated is the use of mAbs produced by cloning and transferring the nucleic acid cloned from a hybridoma of this invention. That is, the nucleic acid expressing the molecules secreted by a hybridoma of this invention can be transferred into another cell line to produce a transformant. The transformant is genotypically distinct from the original hybridoma but is also capable of producing antibody molecules of this invention, including immunologically active fragments of whole antibody molecules, corresponding to those secreted by the hybridoma. See, for example, U.S. Pat. No. 4,642,334 to Reading; PCT Publication No.; European Patent Publications No. 0239400 to Winter et al. and No. 0125023 to Cabilly et al.

In a particular embodiment, mAbs recognizing PD-L1 may be generated by immunization of Balb-c mice with the respective recombinant human Fc-IgGl fusion proteins. Spleen cells were fused with X-63 myeloma cells and cloned according to already described procedures (Olive D, 1986). Hybridoma supernatants were then screened by staining of transfected cells and for lack of reactivity with untransfected cells.

Antibody generation techniques not involving immunisation are also contemplated such as for example using phage display technology to examine naive libraries (from non-immunised animals); see (Barbas et al, 1992, and Waterhouse et al. (1993). Antibodies of the invention are suitably separated from the culture medium by conventional immunoglobulin purification procedures such as, for example, affinity, ion exchange and/or size exclusion chromatography, and the like.

In a particular embodiment, the antibody of the invention may be a human chimeric antibody. Said human chimeric antibody of the present invention can be produced by obtaining nucleic sequences encoding VL and VH domains, constructing a human chimeric antibody expression vector by inserting them into an expression vector for animal cell having genes encoding human antibody CH and human antibody CL, and expressing the expression vector by introducing it into an animal cell. The CH domain of a human chimeric antibody may be any region which belongs to human immunoglobulin, but those of IgG class are suitable and any one of
subclasses belonging to IgG class, such as IgG1, IgG2, IgG3 and IgG4, can also be used. Also, the CL of a human chimeric antibody may be any region which belongs to Ig, and those of kappa class or lambda class can be used. Methods for producing chimeric antibodies involve conventional recombinant DNA and gene transfection techniques are well known in the art (See Morrison SL. et al. (1984) and patent documents US5,202,238; and US5,204, 244).

In another particular embodiment, said antibody may be a humanized antibody. Said humanized antibody may be produced by obtaining nucleic acid sequences encoding for CDRs domain by inserting them into an expression vector for animal cell having genes encoding a heavy chain constant region identical to that of a human antibody; and a light chain constant region identical to that of a human antibody, and expressing the expression vector by introducing it into an animal cell. The humanized antibody expression vector may be either of a type in which a gene encoding an antibody heavy chain and a gene encoding an antibody light chain exist on separate vectors or of a type in which both genes exist on the same vector (tandem type). In respect of easiness of construction of a humanized antibody expression vector, easiness of introduction into animal cells, and balance between the expression levels of antibody H and L chains in animal cells, a tandem type of the humanized antibody expression vector is more preferable (Shitara K et al. 1994). Examples of the tandem type humanized antibody expression vector include pKANTEX93 (WO 97/10354), pEE18 and the like. Methods for producing humanized antibodies based on conventional recombinant DNA and gene transfection techniques are well known in the art (See, e.g. Riechmann L. et al. 1988; Neuberger MS. et al. 1985). Antibodies can be humanized using a variety of techniques known in the art including, for example, CDR-grafting (EP 239,400; PCT publication WO91/09967; U.S. Pat. Nos. 5,225,539; 5,530,101; and 5,585,089), veneering or resurfacing (EP 592,106; EP 519,596; Padlan EA (1991); Studnicka GM et al. (1994); Roguska MA. et al. (1994)), and chain shuffling (U.S. Pat. No.5,565,332). The general recombinant DNA technology for preparation of such antibodies is also known (see European Patent Application EP 125023 and International Patent Application WO 96/02576).

Preferably, the anti-PD-L1 antibody fragments are chosen from Fab (e.g., by papain digestion), Fab' (e.g., by pepsin digestion and partial reduction), F(ab)2, F(ab')2 (e.g., by pepsin digestion) and dAb fragments.
Such fragments may be produced by enzymatic cleavage, synthetic or recombinant techniques, as known in the art and/or as described herein. Antibodies can also be produced in a variety of truncated forms using antibody genes in which one or more stop codons have been introduced upstream of the natural stop site. The various portions of antibodies can be joined together chemically by conventional techniques, or can be prepared as a contiguous protein using genetic engineering techniques.

Said Fab fragment of the present invention can be obtained by treating an antibody which specifically reacts with human PD-L1 with a protease, papaine. Also, the Fab may be produced by inserting DNA encoding Fab of the antibody into a vector for prokaryotic expression system or for eukaryotic expression system, and introducing the vector into a procaryote or eucaryote to express the Fab.

Said F(ab')2 of the present invention may be obtained by treating an antibody which specifically reacts with PD-1 with a protease, pepsin. Also, the F(ab')2 can be produced by binding Fab' described below via a thioether bond or a disulfide bond.

Said Fab' may be obtained by treating F(ab')2 which specifically reacts with PD-1 with a reducing agent, dithiothreitol. Also, the Fab' can be produced by inserting DNA encoding Fab' fragment of the antibody into an expression vector for prokaryote or an expression vector for eukaryote, and introducing the vector into a prokaryote or eukaryote to effect its expression.

In a particular embodiment, the anti-PD-L1 antibody derivatives are chosen from scFv, (scFv)2, diabodies, multimeric scFv derived from an anti-PD-L1 antibody and fused to a Fc fragment, whole anti-PD-L1 antibodies linked together to reach an aggregated form, and antibodies containing at least two Fabs bound face-to-tail.

Said scFv fragment may be produced by obtaining cDNA encoding the VH and VL domains as previously described, constructing DNA encoding scFv, inserting the DNA into an expression vector for prokaryote or an expression vector for eukaryote, and then introducing the expression vector into a prokaryote or eukaryote to express the scFv. To generate a humanized scFv fragment, a well-known technology called CDR grafting may be used, which involves selecting the complementary determining regions (CDRs) from a donor scFv fragment, and grafting them onto a human scFv fragment framework of known three dimensional
structure (see, e.g., W098/45322; WO 87/02671; US5,859,205; US5,585,089; US4,816,567; EP0173494).

In a particular embodiment, the anti PD-L1 antibody according to the invention is a monoclonal antibody.

Several monoclonal anti-PD-L1 antibodies have been developed in the art and are available to the skilled artisan.

Suitable monoclonal anti-PD-L1 antibodies that can be used to produce an anti-PD-L1 immunotoxin according to the present invention, include, but are not limited to the monoclonal antibodies described and characterized in Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunes JA, et al. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. International immunology. 2010;22:651-60, namely:

- the PD-L1.1 antibody, obtainable from hybridoma deposited at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on October 15, 2008 under deposited number CNCM 1-4080.

- the PD-L1.2 antibody, obtainable from hybridoma deposited at the Collection Nationale de Cultures de Microorganismes (CNCM, Institut Pasteur, 25 rue du Docteur Roux, 75724 Paris Cedex 15, France), in accordance with the terms of Budapest Treaty, on October 15, 2008 under deposited number CNCM 1-4081.

- the PD-L1.3 antibody, also called PD-L1.3.1 antibody having the CDR sequences described below.

Also available are the anti-PD-L1 antibodies which are commercialized under the following references: 29E.2A3 (biolegend), MIH1(ebioscience, becton Dickinson) and 2E3 (origene).
Advantageously, the anti-PD-L1 antibody of the present invention has the following characteristics:

- immunoreactivity is retained after radio-labeling,
- affinity for PD-L1 is high (typically, a Kd value of at least 10nM, preferably a Kd value around 1.0 nM).
- internalization upon binding of the antibody to the tumor cell.

This is a favorable characteristic for imaging, since the detectable moiety (e.g.) ¹¹¹In-DTPA is trapped in the tumor cell after internalization and degradation of the antibody (26). This results in enhanced tumor-to-background contrast.

In a particular embodiment, the anti-PD-L1 antibody used to produce the immunotoxin of the invention is an antibody that is internalized by tumor cells.

Internalization can be assessed according to any suitable method: flow cytometry (loss of PD-L1 expression), microscopy or western blot (protein disappearance. Typically, an anti-PD-L1 antibody is deemed to be internalized by tumor cells if it leads to cell death according to the in vitro test A described below.

Test A (internalization of the anti-PD-L1 antibody): Briefly, the prostate cell line PC3 is incubated with the anti-PD-L1 antibody with a range from 25 nM to 0.01 nM and a saporin-conjugated goat anti-mouse antibody IgG secondary antibody (referred to as Mab-ZAP, 50 ng) or a negative control saporin-conjugated pre-immune goat IgG (Ig-SAP, 50 ng) for 48 hours at 37°C. The anti-PD-L1 antibody/saporin complex is bound by the targeted cells positive for PD-L1 expression, internalized, and saporin is released to inactivate ribosomes. Cell death is then measured by any suitable method. Typically, cell death can be measured by measuring caspase activity using the Promega caspase Glow 3/7 assay luminescence kit.

In one embodiment the anti-PD-L1 antibody according to the present invention is selected in the group consisting of the monoclonal antibody PD-L1.3.1,
antibodies having the same CDRs as PD-L1.3.1, fragments and derivatives thereof having the above mentioned characteristics.

As used herein, the terms "PD-L1.3.1" and "PD-L1.3" are used interchangeably and refer to the murine monoclonal antibody developed by the inventors and characterized in Ghiotto M, Gauthier L, Serriari N, Pastor S, Truneh A, Nunes JA, et al. PD-L1 and PD-L2 differ in their molecular mechanisms of interaction with PD-1. International immunology. 2010;22:651-60.

The 6 CDRs of the PD-L1.3.1 antibody are as in Table 1 below:

<table>
<thead>
<tr>
<th>CDR</th>
<th>DNA sequence</th>
<th>Aminoacid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-CDR1</td>
<td>GACACCTATATGCAC (SEQ ID NO:1)</td>
<td>DTYMH (SEQ ID NO:7)</td>
</tr>
<tr>
<td>H-CDR2</td>
<td>TGGATGGATCTCTGGAATGAAATAC (SEQ ID NO:2)</td>
<td>WIDFANGNTKYDPKFGQ (SEQ ID NO:8)</td>
</tr>
<tr>
<td>H-CDR3</td>
<td>TCTGGGGTTAGTGCGGCACTTGTA (SEQ ID NO:3)</td>
<td>SGVSTAHFDY (SEQ ID NO:9)</td>
</tr>
<tr>
<td>L-CDR1</td>
<td>AGGGCCAGCTCAAGTGTAAGTTCATGCAC (SEQ ID NO:4)</td>
<td>RASSSVSFMH (SEQ ID NO:10)</td>
</tr>
<tr>
<td>L-CDR2</td>
<td>GCCACATCCACCTCCTTTCTCTC (SEQ ID NO:5)</td>
<td>ATSNLAS (SEQ ID NO:11)</td>
</tr>
<tr>
<td>L-CDR3</td>
<td>CGACGTTGAGTAGTTCAACGCCGACG (SEQ ID NO:6)</td>
<td>QQWSSYPT (SEQ ID NO:12)</td>
</tr>
</tbody>
</table>

*Table 1*

The complete sequences of the variable regions (VH and VL) of the PD-L1.3.1 mAb are the following:

**Heavy chain: DNA sequence (414 bp):**

```
ATGAAAAATGCAGCTGGGGTTATCTTCTCTTCTATGGCAGTGGTTACAGGGGTCA
ATTCCAGGTTGTCAGCTGACAGTCTGGGACAGAACTTGTTGAGCCAGGGGGGCTCAG
TCAAGTTGTCCTGC ACAAATCTGGGCTCCACACTCA AGACACCTATAATGGCTTGGG
TGAAGGCAGGCTGGAACAGGGGCCCTGGAGATGGATGGAGTGGATGAGTCCCTGCAGATG
GAAATACCAAAT ATGACCCGCACTTCTCCTCTGGGGCTGGAAATACGAGGGACCCTCTT
CTCCACACAGCCTACCCGCAGCTCCGCCGGCCCTGACATCTGAGGACACTGCGCCGCTCT
```
ATTACTGTGCTAGATCTGGGTTAGTACGCCCACCTTGACTAC TGGGGCCAAGGCA CCACTCTCACAGTCTCCTCA (SEQ ID NO: 13)

Heavy chain: Amino acids sequence (138 AA): Leader sequence- FR2-COR2-FR3-COR3-FR4
5 MKC SWVI FFLMAWTGVNS EVQLQQSG TELVKGASVKL SCTTSGFNIQDTY MRWVKQRPEQGLEWI GA IDPANGNTKYDPKFQG KATI1ADTSTNAYLQRLTSED TAVYYCAR SG STARFOY WQGTTTVSS (SEQ ID NO:14)

Light chain: DNA sequence (384 bp): Leader sequence- CDR2-LEADER-CDR3-FR3-COR3-FR4

ATGGATTTTCAAGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCTTCAGTCA

The present invention also refers to antibodies comprising SEQ ID NO: 18 in their heavy chain and SEQ ID NO:20 in their light chain. It also refers to antibodies encoded by at least nucleotidic sequences SEQ ID NO: 17 for the heavy chain and SEQ ID NO: 19 for the light chain:

Heavy chain: DNA sequence: FR1-COR1-LEADER-COR2-LEADER-COR3-LEADER-COR4

GAGGTTCAGCTGACAGATTTTCAGCTTCCTGCTAATCAGTGCTTCAGTCA

Light chain: Amino acids sequence (128 AA): Leader sequence- FR2-COR2-FR3-COR3-FR4

MDFQVQIFSFLLISLAVMSRGQI VLSQSPAILSAASPGKVTMTCRASSVS FMRWYQQKPGSSSKPWI YAT SNL ASGVTRFSSGSGTSYSTLSRVEAEDAAANYC QOWSSYFRT FGGTKLEIK (SEQ ID NO:16)
ATTAcTgTgcTAGtctgggttagtacggccccactttgactac tggggccaaaggca ccactctcacagtctctccta (SEQ ID NO:17)

Heavy chain: Amino acids sequence: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4

EVQLQQSGTELVKPGASVKLSCTTSGFNIQOTYmWVKQRPEQGLEWIGmO ___ PANGNTKYOPKFQGKAT I IADTSNTAYLQLRGLTSED TAVYYCAR SGVSTARFOYW GQGTTTLIVSS (SEQ ID NO:18)

Light chain: DNA sequence: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4

CAAAATGGTCTCCTCAGCTCAGCAATCTGTCTGCA TCTCCAGGGGAGA AGGTCACAAATGACTTG CAGGGCCAGCTCAAGTGAAGTCAATGAC TGTACCAGC AGAAGGCCGGATCCTCCCCAAAACCTGGGATT A TGCACATCCAAACCTGGCTTCT G GATCCCCCTACTCGCTTCAGTGCGACGGTGGTCTGGAACCTCTTACTCTCACA CTCA

Light chain: Amino acids sequence: FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4

QIVLQSPAILSAASPGEKVMT CRASSSVSFMRWYQQKPGSSPKPI YATS N1AS GVTRFSGSGSGTSYSLTL SRVEAEDATY YCQQWSSYPRT FGGGTKLEI K (SEQ ID NO:20)

In one embodiment, the present invention relates to a labelled anti-PD-L1 antibody, having the following 6 CDRs:

<table>
<thead>
<tr>
<th></th>
<th>Aminoacid sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>H-CDR1</td>
<td>DTYMH (SEQ ID NO:7)</td>
</tr>
<tr>
<td>H-CDR2</td>
<td>WIDPANGNTKYD PKFQG</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO:8)</td>
</tr>
<tr>
<td>H-CDR3</td>
<td>SGVSTAHFDY</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO:9)</td>
</tr>
<tr>
<td>L-CDR1</td>
<td>RASSSVSFMH</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO:10)</td>
</tr>
<tr>
<td>L-CDR2</td>
<td>ATSNLAS</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO:11)</td>
</tr>
<tr>
<td>L-CDR3</td>
<td>QQWSSYPRT</td>
</tr>
<tr>
<td></td>
<td>(SEQ ID NO:12)</td>
</tr>
</tbody>
</table>
The anti-PD-L1 antibody according to the present invention is labelled with a detectable moiety, thereby enabling the detecting of PD-L1-positive tumors by imaging.

Appropriate detectable moieties or labels according to the present invention are known in the art and can readily be selected by the skilled person in the art. In a particular embodiment, the anti-PD-L1 antibody is labelled with a detectable moiety selected from radionuclides and fluorophores.

Suitable imaging techniques according to the present invention can be gamma imaging and Single Photon Emission Computed Tomography (SPECT) or positron emission tomography (PET) or magnetic resonance imaging (MRI) or fluorescence spectroscopy or optical imaging methods.

Typically, the anti-PD-L1 antibody can be labelled with a radionuclide selected in the group consisting of $^{11}$C, $^{13}$N, $^{15}$O, $^{18}$F, $^{124}$I, $^{68}$Ga, $^{62}$Cu, $^{64}$Cu, $^{99m}$Tc, $^{111}$In and $^{123}$I.

In a particular embodiment, the anti-PD-L1 antibody of the present invention is labelled with $^{111}$In or $^{89}$Zr.

Typically, $^{111}$In is most suitable for imaging by SPECT, whereas $^{89}$Zr is most suitable for PET.

As used herein, the term "tumor" refers to an abnormal mass or population of cells that result from excessive cell division, whether malignant or benign, and all pre-cancerous and cancerous cells and tissues. A tumor can be solid tumor or a haematological malignancy.

Examples of solid tumors include, but are not limited, to prostate cancer, pancreatic cancer, breast cancer, melanoma, B cell lymphoma, brain cancer, bladder cancer, colon cancer, intestinal cancer, lung cancer, stomach cancer, cervical cancer, ovarian cancer, liver cancer, skin cancer, colorectal cancer, endometrial carcinoma,
salivary gland carcinoma, kidney cancer, thyroid cancer, various types of head and neck cancers.

In one embodiment of the invention, said solid tumor is selected among prostate, breast and pancreatic cancers.

In another embodiment, the tumor is a hematological malignancy selected from B-cell lymphoid neoplasm, T-cell lymphoid neoplasm, non-Hodgkin lymphoma (NHL), B-NHL, T-NHL, chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL), mantle cell lymphoma (MCL), NK-cell lymphoid neoplasm and myeloid cell lineage neoplasm.

As used herein, the term "subject" denotes a mammal, such as a rodent, a feline, a canine, and a primate. Preferably a subject according to the invention is a human.

According to the invention, the term "patient" or "patient in need thereof" is intended for a human or non-human mammal affected or likely to be affected by a tumor.

As used herein, the term "PD-L1-positive tumor" denotes a tumor which expresses PD-L1 and is therefore likely to respond to a therapy which targets PD-L1 or its ligand PD-1.

In another aspect, the present invention relates to a method for detecting a PD-L1-positive tumor in a patient who has been administered a labelled anti-PD-L1 antibody, said method comprising subjecting the patient to imaging.

The labelled anti-PD-L1 antibody can be formulated with a pharmaceutically acceptable carrier, preferably for in a form suitable for administration by intravenous injection.

"Pharmaceutically" or "pharmaceutically acceptable" refers to molecular entities and compositions that do not produce an adverse, allergic or other untoward reaction when administered to a mammal, especially a human, as appropriate. A pharmaceutically acceptable carrier or excipient refers to a non-toxic solid, semi-
solid or liquid filler, diluent, encapsulating material or formulation auxiliary of any type.

The form of the pharmaceutical compositions, the route of administration, the dosage and the regimen naturally depend upon the condition to be treated, the severity of the illness, the age, weight, and sex of the patient, etc.

The pharmaceutical compositions of the invention can be formulated for a topical, oral, intranasal, intraocular, intravenous, intramuscular or subcutaneous administration and the like.

Preferably, the pharmaceutical compositions contain vehicles which are pharmaceutically acceptable for a formulation capable of being injected. These may be in particular isotonic, sterile, saline solutions (monosodium or disodium phosphate, sodium, potassium, calcium or magnesium chloride and the like or mixtures of such salts), or dry, especially freeze-dried compositions which upon addition, depending on the case, of sterilized water or physiological saline, permit the constitution of injectable solutions.

The doses used for the administration can be adapted as a function of various parameters, and in particular as a function of the mode of administration used, of the relevant pathology, or alternatively of the desired duration of treatment.

To prepare pharmaceutical compositions, an effective amount of labelled anti-PD-L1 antibody may be dissolved or dispersed in a pharmaceutically acceptable carrier or aqueous medium.

The pharmaceutical forms suitable for injectable use include sterile aqueous solutions or dispersions; formulations including sesame oil, peanut oil or aqueous propylene glycol; and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersions. In all cases, the form must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms, such as bacteria and fungi.

Solutions of the active compounds as free base or pharmacologically acceptable salts can be prepared in water suitably mixed with a surfactant, such as hydroxypropylcellulose. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, mixtures thereof and in oils. Under ordinary conditions of
storage and use, these preparations contain a preservative to prevent the growth of microorganisms.

The carrier can also be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetables oils. The proper fluidity can be maintained, for example, by the use of a coating, such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. The prevention of the action of microorganisms can be brought about by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars or sodium chloride.

Sterile injectable solutions are prepared by incorporating the active compounds in the required amount in the appropriate solvent with various of the other ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the various sterilized active ingredients into a sterile vehicle which contains the basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum-drying and freeze-drying techniques which yield a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

The preparation of more, or highly concentrated solutions for direct injection is also contemplated, where the use of DMSO as solvent is envisioned to result in extremely rapid penetration, delivering high concentrations of the active agents to a small tumor area.

Upon formulation, solutions will be administered in a manner compatible with the dosage formulation and in such amount as is effective for imaging. The skilled person can readily determine the dose of anti-PD-L1 antibody that is required in order to obtain the best signal-to-noise ratio, depending on the detectable moiety and the imaging technique.

For instance, the inventors have found that optimal contrast was obtained at antibody protein doses inferior or equal to 1 microgram of PD-L1.3.1. This optimum dose will be dependent on each specific labelled anti-PD-L1 antibody and can easily
be determined by the skilled artisan using routine experimentation for imaging set-ups.

Similarly, the optimal timing of the imaging with respect to the injection time point can easily be determined by the skilled artisan. In the Examples below, the inventors have found that the optimal time-point for imaging was around 3 to 7 days post-injection.

The present invention also relates to a method for detecting a PD-L1-positive tumor in a patient comprising the steps consisting of:
- administering to said patient a labelled anti-PD-L1 antibody,
- subjecting said patient to imaging,
wherein the patient is considered to suffer from a PD-L1-positive tumor if a positive signal is detected by imaging.

The labelled anti-PD-L1 antibody according to the invention is useful for detecting PD-L1-positive tumors and can therefore be used in methods for predicting the response to treatment which targets PD-L1 or its ligand PD-1. Typically, the labelled anti-PD-L1 antibody of the invention can be used to select patients for a treatment with anti-PD-1 or anti-PD-L1 antibodies, such as those described in WO 2010/089411 or in references (6-11) below.

Without wishing to be bound by theory, the inventors believe that molecular imaging with radiolabeled anti-PD-L1 antibodies according to the present invention has important advantages over immunohistochemical analysis of PD-L1 expression. First of all, imaging allows measurement of PD-L1 expression of whole tumor lesions and their metastases, thereby avoiding sampling errors and thus misinterpretation due to intratumoral and interlesional heterogeneity. Second, it allows monitoring of expression during the course of disease, without the need of repetitive biopsies. This is of clinical importance since PD-L1 expression can change during the course of disease, due to disease progression and/or the effects of treatment^\textsuperscript{15, 18, 19} Third, in vivo imaging techniques also take into account the in vivo target accessibility for agents that are administered systemically. Several factors may determine whether antibodies will reach the tumor cells, such as blood vessel
density, vascular permeability and interstitial fluid pressure. (24, 25) Therefore, in vivo imaging with radiolabeled anti-PD-L1 antibodies provides more relevant information about PD-L1 expression and accessibility than immunohistochemistry, it allows patient selection for PD-1/PD-L1 targeted therapy, and can be used to monitor PD-L1 expression during the course of disease.

In another aspect, the invention relates to a method for monitoring the response to treatment in a patient suffering from cancer comprising the steps consisting of:
- carrying out the above method at a first time point t1;
- carrying out the above method at a second time point t2;
wherein a decrease in the expression level of PD-L1 observed at t2 compared to t1 is indicative of a response to treatment.

In yet another aspect, the invention relates to a method for treating cancer in a patient comprising the steps of:
- detecting a PD-L1-positive tumor in said patient by the above method;
- if the patient is considered to suffer from a PD-L1-positive tumor, administering an anti-PD1 or anti-PD-L1 antibody to said patient in a therapeutically effective amount.

The invention will be further illustrated through the following examples and figures.

**FIGURES LEGENDS**

**Figure 1.** A: Binding of $^{111}$In-PD-L 1.3.1 to five different breast cancer cell lines. B: IC50 analysis of PD-L1.3.1. C: Scatchard analysis of $^{111}$In-PD-L 1.3.1. D: Internalization kinetics of $^{111}$In-PD-L 1.3.1.

**Figure 2.** A: Dose escalation study of $^{111}$In-PD-L 1.3.1 in mice with subcutaneous MDA-MB-231 xenografts, 3 days p.i. B: Tumor uptake of $^{111}$In-PD-L 1.3.1 (1 µg) in
mice with subcutaneous MDA-MB-23 1 or MCF-7 xenografts. Separate groups of mice were injected with an excess of unlabeled PD-L1.3.1.

**Figure 3.** A: Typical examples of SPECT/CT scans of mice with subcutaneous MDA-MB-23 1 or MCF-7 xenografts, acquired at different time-points after injection of 15.5 MBq $^{111}$In-PD-L1.3.1 (1.5 µg). Tumors are indicated with the white arrows. B: Close-up of the heterogeneous targeting of $^{111}$In-PD-L1.3.1 in MDA-MB-23 1 xenografts. C: Uptake of $^{111}$In-PD-L1.3.1 in the tumor and liver of mice bearing subcutaneous MDA-MB-23 1 or MCF-7 xenografts, as quantified from the SPECT scans.

**Figure 4.** SPECT/CT scans of mice bearing subcutaneous breast cancer xenografts, with different PD-L1 expression levels, on both flanks (indicated with the white arrows). Scans were acquired three days post injection of 10 MBq $^{111}$In-PD-L1.3.1 (1 µg).

**Figure 5.** Examples of autoradiography and cross section of SPECT scans to illustrate the heterogeneous distribution of $^{111}$In-PD-L1.3.1 in the xenograft, HE staining, and PD-L1 immunostaining of breast cancer xenografts.

**EXAMPLES**

**Material and methods**

**Cell culture**

The breast cancer cell lines MDA-MB-23 1, SK-Br-3, and MCF-7 were cultured in RPMI1640 (GIBCO, BRL Life Sciences Technologies, The Netherlands), supplemented with 2 mM glutamine (GIBCO) and 10% FCS (Sigma-Aldrich Chemie BV, The Netherlands) at 37 °C in a humidified atmosphere with 5% CO$_2$. SUM149 was cultured in Ham's F12 medium (GIBCO) supplemented with 5% FCS, 10 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES, GIBCO), hydrocortisone (1 µg/ml, Sigma-Aldrich Chemie BV), and insulin (5 µg/ml, Sigma-Aldrich Chemie BV). BT474 was cultured in RPMI1640, 2 mM glutamine, 10% FCS, and 10 µg/ml insulin.

**FACS analysis of PD-L1 expression**
PD-L1 expression of MDA-MB-231, SK-Br-3, SUM149, BT474, and MCF-7 cells was determined by FACS analysis. Cells were incubated with PD-L1-PE (557924, BD biosciences, San Jose, CA) or mouse IgGl-PE (4001 14, Biolegend, San Diego, CA) for 30 min at 4°C. Cells were washed and, subsequently, analyzed using the Gallios flow cytometer (Beckman Coulter, Fullerton, CA, USA).

**Radiolabeling**

The murine monoclonal IgGl antibody PD-L 1.3.1 is specifically directed against human PD-L1 and does not cross react with murine PD-L1.(21) It was conjugated with isothiocyanatobenzyl-diethyleneetriaminepentaacetic acid (ITC-DTPA, Macrocyclis, Dallas, TX) in 0.1 M NaHCO₃, pH 9.5, at a 21-fold molar excess of ITC-DTPA, for 1 h at room temperature (RT). Unbound ITC-DTPA was removed from the reaction mixture by dialysis against 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES, Sigma-Aldrich Chemie BV) buffer, followed by purification on a disposable G25M Sephadex column (PD10, GE Healthcare Life Sciences, Eindhoven, The Netherlands), eluted with 0.25 M ammoniumacetate buffer, pH 5.4 (Sigma-Aldrich Chemie BV).

DTPA-conjugated PD-L 1.3.1 was incubated with ¹¹¹In (Mallinckrodt BV, Petten, The Netherlands) in 0.5 M MES buffer, pH 5.4, 20 min at RT, under strict metal-free conditions.(22) After incubation, 50 mM ethylenediammetetraacetic acid (EDTA) was added to a final concentration of 5 mM to chelate unincorporated ¹¹¹In. Labeling efficiency was determined using instant thin-layer chromatography (ITLC) on silica gel chromatography strips (Agilent Technologies, Palo Alto, CA), using 0.1 M citrate buffer (Sigma-Aldrich Chemie BV), pH 6.0, as the mobile phase. In case labeling efficiency was below 95%, the reaction mixture was purified on a PD-10 column, eluted with PBS, containing 0.5% BSA (Sigma-Aldrich Chemie BV). Radiochemical purity of ¹¹¹In-DTPA-PD-L 1.3.1 (¹¹¹In-PD-L 1.3.1) exceeded 95% in all experiments.

**In vitro assays**

**Binding to breast cancer cell lines**

The breast cancer cell lines MDA-MB-231, SK-Br-3, SUM149, BT474, and MCF-7 were cultured to confluency in six-well plates and incubated with 32 pM ¹¹¹In-PD-L 1.3.1 (1 kBq) for 4 h at 37 °C in a humified atmosphere with 5% CO₂, or for 4 h on ice, in RPMI1640 containing 0.5% BSA. Separate wells were coincubated
with a 1,000-fold excess of unlabeled PD-L 1.3.1 to determine non-specific binding. After incubation, cells were washed with PBS and the cell-associated activity was measured in a shielded well-type gamma counter (Perkin-Elmer, Boston, MA, USA). Specific binding was calculated by subtracting the non-specific binding from the total binding.

**Immunoreactive fraction**

The immunoreactive fraction (IRF) of $^{111}$In-PD-L 1.3.1 was determined essentially as described by Lindmo et al (26). A serial dilution of MDA-MB-231 cells (3.3x10^5 - 8.4x10^7 cells/ml) in RPMI1640 containing 0.5% BSA was incubated with 8 pM $^{111}$In-PD-L 1.3.1 (0.2 kBq). Non-specific binding was determined by adding an excess of unlabeled PD-L 1.3.1 (67 nM) to a duplicate of the lowest cell concentration. After 1 h incubation at 37 °C, cells were centrifuged and the activity in the cell pellet was measured in a shielded 3-inch-well-type gamma counter (Perkin-Elmer, Boston, MA, USA). The inverse of the specific cell bound activity was plotted against the inverse of the cell concentration, and the immunoreactive fraction was calculated from the y-axis intercept using GraphPad Prism (version 5.03 for Windows).

**IC$_{50}$**

MDA-MB-231 cells were cultured to confluence in six-well plates. The 50% inhibitory concentration (IC$_{50}$) of PD-L 1.3.1 blocking of $^{111}$In-PD-L 1.3.1 binding was determined by incubating the cells for 4 h on ice in 1 ml RPMI1640 0.5% BSA, containing 20 pM $^{111}$In-PD-L 1.3.1 (1 kBq) and increasing concentrations of unlabeled PD-L 1.3.1 (1 - 1000 pM). After incubation, cells were washed with PBS and the cell-associated activity was measured in a gamma counter. The IC$_{50}$ was defined as the antibody concentration that was required to inhibit binding of the radiolabeled antibody by 50%. IC$_{50}$ values were calculated using GraphPad Prism.

**Scatchard analysis**

Scatchard analysis was performed to determine the dissociation constant (K$_d$) of $^{111}$In-PD-L 1.3.1 and to quantitatively measure PD-L1 expression on MDA-MB-231, SK-Br-3, and SUM149 cells. Cells were cultured to confluence in six-well plates and were incubated for 4 h on ice with increasing concentrations $^{111}$In-PD-L1.3.1 (3 - 3000 pM) in 1 ml RPMI1640 containing 0.5% BSA. Non-specific binding was determined by co-incubation with 100 nM PD-L 1.3.1. After incubation,
cells were washed with PBS and the cell-associated activity was measured in a
shielded well-type gamma counter. The specific binding (total binding - nonspecific
binding) was plotted against the bound/free ratio. Data were analyzed by linear
regression to determine PD-L1 receptor density per cell and to determine the 3/4 of

\[ 1^{11} \text{In-PD-L1.3.1} \]

Internalization kinetics

MDA-MB-231 cells were cultured in six-well plates and were incubated for
2, 4, or 24 h with 75 pM \[ 1^{11} \text{In-PD-L1.3.1} \] (1 kBq) in RPMI1640 containing 0.5%
BSA at 37 °C in a humidified atmosphere with 5% CO\(_2\). Nonspecific binding and
internalization was determined by coincubation with 17 nM unlabeled PD-L1.3.1.
After incubation, acid wash buffer (0.1 M HAc, 0.15 M NaCl, pH 2.6) was added for
10 min to remove the membrane-bound fraction of the cell-associated \[ 1^{11} \text{In-PD-}
L1.3.1 \]. Subsequently, cells were harvested from the six-well plates and the amount
of membrane bound and internalized activity was measured in a gamma counter.

Specific binding and internalization were calculated by subtracting the non-specific
binding and internalization from the total binding and internalization.

Animal studies

Animal experiments were performed on female BALB/c nude mice (Janvier,
le Genest-Saint-Isle, France) and were conducted in accordance with the principles
laid out by the revised Dutch Act on Animal Experimentation (1997) and approved
by the institutional Animal Welfare Committee of the Radboud University Nijmegen.
At 6-8 weeks of age, mice were inoculated subcutaneously with 5 x 10^6 MDA-MB-
231, SK-Br-3, SUM149, MCF-7, or BT474 cells (mixed 2:1 with matrigel, BD
Biosciences, Pharmingen). Mice receiving MCF-7 or BT474 cells were, prior to
tumor cell inoculation, implanted subcutaneously with a slow release estradiol pellet
(0.18 mg, 60 days, Innovative Research of America, Sarasote, FL) under general
anesthesia (isoflurane/0.2). Experiments started when tumors reached a size of
approximately 0.1 cm\(^3\).

Dose optimization

Seven groups (n=6) of mice with subcutaneous MDA-MB-231 xenografts
received an intravenous injection of 0.2 MBq \[ 1^{11} \text{In-PD-L1.3.1} \] (specific activity 0.4
MBq/g) in the tail vein. To study the effect of the antibody protein dose on the
biodistribution of \[ 1^{11} \text{In-PD-L1.3.1} \], groups received increasing protein doses of PD-
LI. 3.1 (0.3 - 300 µg/mouse). Three days post injection, mice were euthanized using C02/O2-asphyxiation. The biodistribution of the radiolabel was determined ex vivo. Tumor, blood, muscle, lung, heart, spleen, pancreas, intestine, kidney, liver, bone, and bone marrow were dissected and weighed. Activity was measured in a gamma counter. To determine the uptake of radiolabeled antibodies in each sample as a fraction of the injected dose, aliquots of the injected dose were counted simultaneously. The results were expressed as percentage injected dose per gram tissue (%ID/g).

Biodistribution studies

Three groups (n=6) of mice with subcutaneous MDA-MB-23 1 xenografts and three groups with MCF-7 xenografts received an intravenous injection of 0.2 MBq 111In-PD-L1 1.3.1. Separate groups of mice were coinjected with an excess of 300 µg unlabeled PD-L1 3.1 to block PD-L1 in vivo. At 1, 3 and 7 days post injection of radiolabeled PD-L1 1.3.1, mice were euthanized and the ex vivo biodistribution of radiolabeled PD-L1 1.3.1 was determined as described previously.

SPECT/CT imaging

Three mice with subcutaneous MDA-MB-23 1 and three mice with subcutaneous MCF-7 xenografts received an intravenous injection of 15.5 MBq 111In-PD-L1 1.3.1 (protein dose 1.5 µg). Immediately after injection and 1, 3 and 7 days post injection, images were acquired with the U-SPECT-II/CT (MI Labs, Utrecht, The Netherlands). (23) Mice were scanned under general anesthesia (isoflurane/O2) for 30 - 90 min using the 1.0 mm diameter pinhole mouse high sensitivity collimator tube, followed by a CT scan (spatial resolution 160 µm, 65 kV, 615 µA) for anatomical reference. Scans were reconstructed with MI Labs reconstruction software, using an ordered-subset expectation maximization algorithm, with a voxel size of 0.2 mm. SPECT/CT scans were analyzed and maximum intensity projections (MIPs) were created using the Inveon Research Workplace software (IRW, version 4.1). A 3D volume of interest was drawn around the tumor and uptake was quantified as the percentage injected dose per gram (%ID/g), assuming a tissue density of 1 g/cm³.

111In-PD-L1 SPECT/CT in breast cancer models with different PD-L1 expression levels
Tumor targeting of $^{111}$In-PD-L 1.3.1 to MDA-MB-231, SK-Br-3, SUM149, MCF-7, and BT474 xenografts was determined in 10 mice bearing subcutaneous tumors on both flanks (n=2 mice/4 tumors per xenograft model). Mice received intravenous tail vein injections of 1 µg (10 MBq) $^{111}$In-PD-L 1.3.1 and 3 days later, mice were euthanized by CO$_2$/O$_2$ asphyxiation and SPECT/CT images were acquired for 90 min, as described previously. Subsequently, the ex vivo biodistribution of radiolabeled PD-L 1.3.1 was determined. Tumors were fixed in 4% formalin or frozen at -80 °C for autoradiography and immunohistochemistry.

**Autoradiography**

Frozen and formalin-fixed tumor sections (5 µm) from mice injected with 10 MBq of $^{111}$In-PD-L 1.3.1 (1 µg) were exposed to a Fujifilm BAS cassette 2025 overnight (Fuji Photo Film). Phospholuminescence plates were scanned using a Fuji BAS-1800 II bioimaging analyzer at a pixel size of 50 × 50 µm. Images were analyzed with Aida Image Analyzer software (Raytest).

**Immunohistochemistry**

Frozen tumor sections of MDA-MB-231, SK-Br-3, SUM149, BT474, and MCF-7 were fixed for 10 min in ice cold acetone (-20 °C). Endogenous mouse Ig staining was blocked using a mouse-on-mouse blocking kit (BMK-2202, Vector). Subsequently, sections were incubated with 10 µg/ml PD-L.3.1, followed by incubation with a peroxidase-conjugated rabbit-anti-mouse antibody (P0260, DAKO). Finally, 3-3'-Diaminobenzidine (DAB) was used to visualize peroxidase activity in the sections.

**Statistical analyses**

Statistical analyses were performed using PASW Statistics version 18.0 (Chicago, IL) and GraphPad Prism version 5.03 (San Diego, CA) for Windows. Differences in uptake of radiolabeled PD-L 1.3.1 were tested for significance using the nonparametric Kruskal-Wallis and Mann-Whitney U test. The correlation between tumor uptake measured by SPECT and ex vivo biodistribution was calculated with the Spearman correlation coefficient. A p-value below 0.05 was considered significant.

**Results**
In-PD-L 1.3.1 specifically binds to PD-L1

Flow cytometry analysis showed that the percentage of tumor cells positive for PD-L1 was 89.4%, 2.9%, 8.9%, 0.2%, and 0.1% for MDA-MB-231, SK-Br-3, SUM149, BT474, and MCF-7, respectively. PD-L1.3.1 was labeled with $^{111}$In, obtaining specific activities up to 10 MBq/g antibody. $^{111}$In-PD-L1.3.1 showed the highest binding to MDA-MB-231 cells, while binding to SUM149 and SK-Br-3 was significantly lower (P=0.002). PD-L1 negative BT474 and MCF-7 cells did not show any specific binding of $^{111}$In-PD-L 1.3.1 (Figure 1A). The number of binding sites for PD-L1.3.1 was determined quantitatively with scatchard analysis and was 47,700 ± 2,900 for MDA-MB-231, 2,000 ± 100 for SK-Br-3, and 3,600 ± 400 for SUM149. Based on these results MDA-MB-231 cells were used in subsequent binding assays.

The IRF of $^{111}$In-PD-L 1.3.1 was 82% and the IC$_{50}$ of unlabeled PD-L1.3.1 was 0.15 nM (Figure 1B). Scatchard analysis showed that the affinity of $^{111}$in-labeled PD-L1.3.1 was 0.97 ± 0.15 nM (Figure 1C). PD-L1.3.1 was slowly internalized by MDA-MB-231 cells. After 24 h of incubation, 25% of the cell-associated activity was internalized and 75% was still membrane-bound (Figure 1D). These data demonstrate that $^{111}$In-PD-L 1.3.1 antibody specifically binds to PD-L1 expressing tumor cells.

$^{111}$In-PD-L 1.3.1 accumulates specifically in PD-L1 positive xenografts

The antibody protein dose escalation study showed high and specific tumor accumulation of $^{111}$In-PD-L1.3 (Figure 2A). Tumor uptake in PD-L1 positive MDA-MB-231 was the highest in mice injected with 0.3 or 1 µg of antibody (37.5 ± 12.5 and 35.7 ± 5.8 %ID/g, respectively, Figure 2A). At antibody doses of ≥ 3 µg, tumor uptake significantly decreased (3 µg: 16.9 ± 3.8 %ID/g, p = 0.002). Tumor uptake was the lowest in mice injected with 300 µg PD-L1.3.1 (7.6 ± 1.6 %ID/g). Other organs did not show specific targeting of $^{111}$in-PD-L 1.3.1.

Uptake of $^{111}$In-PD-L 1.3.1 by PD-L1 positive MDA-MB-231 xenografts was observed as early as 1 day post injection and further increased at day 3 and 7 (Table 2, Figure 2B). PD-L1 negative MCF-7 xenografts did not show specific uptake at any time point. Tumor-to-blood ratios increased over time for MDA-MB-231 and were the highest 7 days post injection (3.9 ± 1.0). Tumor-to-blood ratios for MCF-7 did not exceed 0.9 and were not significantly increased compared with the tumor-blood-
ratios in mice that received an excess of unlabeled PD-L1.3.1. These data demonstrate that $^{111}$In-PD-L 1.3.1 can discriminate between PD-L1 positive and PD-L1 negative xenografts.

Table 2. Tumor targeting of $^{111}$In-PD-L1.3.1 to MDA-MB-231 and MCF-7 xenografts at 1, 3, and 7 days post injection.

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<tr>
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<tr>
<td>Day 1</td>
<td>16.8 ± 3.8</td>
<td>1.1 ± 0.2</td>
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<tr>
<td>Day 3</td>
<td>36.4 ± 4.0</td>
<td>3.1 ± 0.2</td>
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<td>Day 7</td>
<td>32.8 ± 6.8</td>
<td>3.9 ± 1.0</td>
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<tr>
<td>Day 3 + excess unlabeled</td>
<td>7.6 ± 1.6</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 1</td>
<td>6.1 ± 0.7</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>Day 3</td>
<td>7.3 ± 1.0</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Day 7</td>
<td>6.2 ± 1.0</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Day 3 + excess unlabeled</td>
<td>7.7 ± 2.2</td>
<td>0.8 ± 0.2</td>
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</table>

SPECT/CT visualizes PD-L1 positive xenografts

MDA-MB-231 xenografts were clearly visualized with $^{111}$In-PD-L1.3.1 SPECT/CT, with increasing contrast between the tumor and normal tissue with time. Typical examples of SPECT/CT scans, and the quantification of tumor and liver uptake are presented in Figure 3. Tumor uptake in MDA-MB-231 xenografts increased over time, while the uptake in MCF-7 xenografts did not exceed uptake in normal organs, such as the liver. Intratumoral distribution of $^{111}$In-PD-L1.3.1 was heterogeneous, as is visualized in a zoomed high-resolution image of the tumor in Figure 3B. There was a strong correlation between the tumor uptake as measured by SPECT and by counting of dissected tissues at 7 days p.i. (Spearman r = 0.94).

$^{111}$In-PD-L1 SPECT/CT can discriminate xenografts with high and low PD-L1 expression levels
SPECT/CT images demonstrated high uptake of $^{111}$In-PD-L 1.3.1 in MDA-MB-231 and SK-Br-3 xenografts and low uptake in SUM149, BT474, and MCF-7 xenografts (Figure 4), which was confirmed in the ex vivo biodistribution study. Tumor uptake at 3 days post injection was 25.2 ± 2.9 %ID/g, 22.0 ± 5.1 %ID/g, 8.4 ± 0.2 %ID/g, 10.0 ± 0.7 %ID/g, and 8.1 ± 1.4 %ID/g, for MDA-MB-231, SK-Br-3, SUM149, BT474, and MCF-7 xenografts, respectively.

Autoradiographical analysis of the tumor sections showed that $^{111}$In-PD-L 1.3.1 antibody was distributed heterogeneously within the tumor. In general, highest tumor uptake was observed in the periphery of the tumor, while the uptake in the tumor center was lower. This heterogeneous distribution was also observed on cross-sections of the SPECT/CT scan (Figure 5). After autoradiographical analysis, the same slides were used for HE staining which showed that the tumors contained areas with vital tissue (mostly in the periphery of the tumor) and areas with necrosis (mostly in the center of the tumor). In MDA-MB-231 and SK-Br-3 xenografts, highest uptake of $^{111}$in-PD-L 1.3.1 was found in the vital part of the tumor.

Immunohistochemical analysis of tumor sections for PD-L1 expression showed that MDA-MB-231 tumors expressed the highest levels of PD-L1. SK-Br-3 xenografts also clearly expressed PD-L1, although the expression levels varied largely between the tumors. Immunostaining of SUM149, BT474, and MCF-7 showed low PD-L1 expression (Figure 5).

In summary, $^{111}$In-PD-L 1.3.1 SPECT/CT can discriminate between xenografts with high and low PD-L1 expression levels.

**Conclusion**

The inventors have shown that the labelled antibodies of the present invention are able to detect PD-L1-positive tumors and to discriminate between tumors having different expression levels of PD-L1.

**REFERENCES**

Throughout this application, various references describe the state of the art to which this invention pertains. The disclosures of these references are hereby incorporated by reference into the present disclosure.


refractory advanced melanoma: a randomised dose-comparison cohort of a phase 1 trial. Lancet. 2014.


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| 0-4-1 | Authorized officer                                                                 | N. Mailliard |</p>
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CLAIMS


2. A labelled anti-PD-L1 antibody for use according to claim 1, wherein said anti-PD-L1 antibody is a monoclonal antibody.

3. A labelled anti-PD-L1 antibody for use according to any of the preceding claims, wherein said antibody is labelled with a detectable moiety selected from radionuclides and fluorophores.

4. A labelled anti-PD-L1 antibody for use according to claim 3, wherein said detectable moiety is a radionuclide selected in the group consisting of $^{11}$C, $^{15}$N, $^{18}$O, $^{18}$F, $^{124}$I, $^{68}$Ga, $^{62}$Cu, $^{64}$Cu, $^{99}$mTc, $^{111}$In and $^{123}$I.

5. A labelled anti-PD-L1 antibody for use according to any of the preceding claims wherein said imaging is performed by SPECT or PET.

6. A labelled anti-PD-L1 antibody for use according to any of the preceding claims wherein said labelled anti-PD-L1 antibody is for administration by intravenous injection.

7. A labelled anti-PD-L1 antibody for use according to any of the preceding claims wherein said tumor is a solid tumor selected from the group consisting of prostate cancer, pancreatic cancer, breast cancer, melanoma, B cell lymphoma, brain cancer, bladder cancer, colon cancer, intestinal cancer, lung cancer, stomach cancer, cervical cancer, ovarian cancer, liver cancer, skin cancer, colorectal cancer, endometrial carcinoma, salivary gland carcinoma, kidney cancer, thyroid cancer, various types of head and neck cancers, preferably breast cancer.
8. A method for detecting a PD-L1-positive tumor in a patient who has been administered a labelled anti-PD-L1 antibody, said method comprising subjecting the patient to imaging.

9. A method for monitoring the response to treatment in a patient suffering from cancer comprising the steps consisting of:
   - carrying out the method according to claim 9 at a first time point t1;
   - carrying out the method according to claim 9 at a second time point t2;
wherein a decrease in the expression level of PD-L1 observed at t2 compared to t1 is indicative of a response to treatment.

10. A method for detecting a PD-L1-positive tumor in a patient comprising the steps consisting of:
   - administering to said patient a labelled anti-PD-L1 antibody,
   - subjecting said patient to imaging,
wherein the patient is considered to suffer from a PD-L1-positive tumor if a positive signal is detected by imaging.

11. A method for treating cancer in a patient comprising the steps of:
   - detecting a PD-L1-positive tumor in said patient by the method according to claim 10;
   - if the patient is considered to suffer from a PD-L1-positive tumor, administering an anti-PDI or anti-PD-L1 antibody to said patient in a therapeutically effective amount.
Figure 1
Figure 3
1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
   a. forming part of the international application as filed:
      - [ ] in the form of an Annex C/ST.25 text file.
      - [ ] on paper or in the form of an image file.
   b. [ ] furnished together with the international application under PCT Rule 13fer1 (a) for the purposes of international search only in the form of an Annex C/ST.25 text file.
   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:
      - [ ] in the form of an Annex C/ST.25 text file (Rule 13fer1 (a)).
      - [ ] on paper or in the form of an image file (Rule 13fer1 (b) and Administrative Instructions, Section 7:3).

2. [ ] In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:
**INTERNATIONAL SEARCH REPORT**

**International application No**

PCT/EP2016/057493

**A. CLASSIFICATION OF SUBJECT MATTER**

INV. A61K51/10 A61P35/00 A61K103/20 C07K16/28 C07K16/30

ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

A61K C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, WPI Data, EMBASE, BIOSIS

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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Further documents are listed in the continuation of Box C. See patent family annex.

"S" Special categories of cited documents:
- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier application or patent but published on or after the international filing date
- "L" later document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed
- "T*" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y*" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "Z" document member of the same patent family

Date of the actual completion of the international search

2 September 2016

Date of mailing of the international search report

13/09/2016

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL-2280 HV Rijswijk
Tel. (+31-70) 340-2040
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Authorized officer

Monami, Amelie
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