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CA-A1- 2 955 541
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Fortsættes ...

EMEA/H/C/003985/0000", EMA/CHMP/76688/2015, 23 April 2015 (2015-04-23), pages 1 - 130, XP093186112
WANG, C. ET AL.: "In Vitro Characterization of the Anti-PD-1 Antibody Nivolumab, BMS-936558, and In Vivo Toxicology in Non-Human Primates", CANCER IMMUNOL RES., vol. 2, 28 May 2014 (2014-05-28), XP055206944

DESCRIPTION

Description

Technical Field

[0001] The present invention relates generally to antibodies of PD-1 and compositions thereof, and immunotherapy in the treatment of human disease using anti-PD-1 antibodies.

Background of the Invention

[0002] Increasing evidences from preclinical and clinical results have shown that targeting immune checkpoints is becoming the most promising approach to treat patients with cancers. The protein Programmed Death 1 (PD-1), an inhibitory member of the immunoglobulin super-family with homology to CD28, is expressed on activated B cells, T cells, and myeloid cells (*Agata et al, supra;* Okazaki et al (2002) Curr. Opin. Immunol. 14: 391779-82*;* Bennett et al. (2003) J Immunol 170:711-8*)* and has a critical role in regulating stimulatory and inhibitory signals in the immune system (Okazaki, Taku et al. 2007 International Immunology 19:813-824*).* PD-1 was discovered through screening for differential expression in apoptotic cells (Ishida et al (1992) EMBO J 11:3887-95*).*

[0003] The PD-1 is a type I transmembrane protein that is part of the Ig gene superfamily (Agata et al. (1996) bit Immunol 8:765-72) and the structure of PD-1 consists of one immunoglobulin variable-like extracellular domain and a cytoplasmic domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Although structurally similar to CTLA-4, PD-1 lacks the MYPPPY motif that is critical for B7-1 and B7-2 binding. PD-1 has two known ligands, PD-L1 (B7-H1, CD274) and PD-L2 (B7-DC, CD273), which are cell surface expressed members of the B7 family (Freeman et al (2000) J Exp Med 192: 1027-34*;* Latchman et al (2001) Nat Immunol 2:261-8*;* Carter et al (2002) Eur J Immunol 32: 634-43*).* Both PD-L1 and PD-L2 are B7 homologs that bind to PD-1, but do not bind to other CD28 family members.

[0004] PD-1, as one of the immune-checkpoint proteins, is an inhibitory member of the CD28 family expressed on activated B cells, T cells, and myeloid cells (*Agata et al, supra;* Okazaki et al. (2002) Curr Opin Immunol 14: 391779-82*;* Bennett et al. (2003) J Immunol 170:711-8) plays a major role in limiting the activity of T cells that provide a major immune resistance mechanism by which tumor cells escaped immune surveillance. PD-1 induces a state of anergy or unresponsiveness in T cells, resulting in the cells being unable to produce optimal levels of effector cytokines. PD-1 may also induce apoptosis in T cells *via* its ability to inhibit survival signals. PD-1 deficient animals develop various autoimmune phenotypes, including autoimmune cardiomyopathy and a lupus-like syndrome with arthritis and nephritis (Nishimura et al. (1999) Immunity 11: 141-51*;* Nishimura et al. (2001) Science 291:319-22). Additionally, PD-1 has been found to play a role in autoimmune encephalomyelitis, systemic lupus erythematosus, graft-versus-host disease (GVHD), type I diabetes, and rheumatoid arthritis (Salama et al. (2003) J Exp Med 198:71-78*:* Prokunina and Alarcon-Riquelme (2004) Hum Mol Genet 13:R143*;* Nielsen et al. (2004) Lupus 11:510*).* In a murine B cell tumor line, the ITSM of PD-1 was shown to be essential to block BCR-mediated Ca^{2+}-flux and tyrosine phosphorylation of downstream effector molecules (Okazaki et al. (2001) PNAS 98: 13866-71).

[0005] The interaction of PD-1 expressed on activated T cells, and PD-L1 expressed on tumor cells negatively regulates immune response and damps anti-tumor immunity. PD-L1 is abundant in a variety of human cancers (Dong et al (2002) Nat. Med 8: 787-9*).* Expression of PD-L1 on tumors is correlated with reduced survival in esophageal, pancreatic and other types of cancers, highlighting this pathway as a new promising target for tumor immunotherapy. Several groups have shown that the PD-1-PD-L interaction exacerbates disease, resulting in a decrease in tumor infiltrating lymphocytes, a decrease in T-cell receptor mediated proliferation, and immune evasion by the cancerous cells (Dong et al. (2003) J. Mol. Med. 81:281-7*;* Blank et al. (2005) Cancer Immunol. Immunother. 54: 307-314*;* Konishi et al. (2004) Clin. Cancer Res. 10:5094-100*).* Immune suppression can be reversed by inhibiting the local interaction of PD-1 with PD-L1, and the effect is additive when the interaction of PD-1 with PD-L2 is blocked as well.

[0006] Anti-PD-1 antibody nivolumab has been characterized in vitro and tested in non-human primates (C. Wang et al. Cancer Immunology Research, vol. 2, no. 9, 28 May 2014, pages 846-856*).* Multiple agents targeting PD-1 pathway have been developed by several pharmaceutical companies, such as Bristol-Myers Squibb (BMS), Merck, Roche and GlaxoSmithKline (GSK). Data from clinical trials demonstrated early evidence of durable clinical activity and an encouraging safety profile in patients with various tumor types. Nivolumab, an anti-PD-1 drug developed by BMS, is being put at center stage of the next-generation field. Now in 6 late-stage studies, the treatment spurred tumor shrinkage in three out of five cancer groups studied, including 18% of lung cancer patients (n=72), close to one third of melanoma patients (n=98) and 27% of patients with kidney cancer (n=33). Developed by Merck, Pembrolizumab is a humanized monoclonal IgG4 antibody that acts against PD-1, which grabbed the FDA's new breakthrough designation after impressive IB data came through for skin cancer. The results from a phase IB study have shown an objective anti-tumor response in 51% of the cancer patients (n=85), and a complete response in 9% of the patients. Roche's experimental MPDL3280A (Atezolizumab)

demonstrated an ability to shrink tumors in 29 of 140 (21%) advanced cancer patients with various tumor sizes.

[0007] Further anti-PD-1 antibodies have been disclosed in WO 2016/077397.

[0008] There are some spaces for improvement for antibody against PD-1 as a therapeutic agent. Most of monoclonal antibodies against PD-1 currently tested in clinical trials are only against to human PD-1 which limits preclinical *in vivo* assay and diminished efficacy owing to the immunogenicity of the mouse-derived protein sequences. Humanized antibody with cross-reactivity to mouse PD-1 overcome these shortages and showed more tolerability and higher efficiency *in vivo.* Thus there is still a need for novel anti-PD-1 antibody.

Disclosure of the Invention

[0009] The present invention is defined by the appended claims.

[0010] The present invention provides isolated antibodies, in particular monoclonal antibodies.

[0011] The present invention provides an antibody or antigen binding fragment thereof that specifically binds to PD-1, wherein the antibody or antigen binding fragment thereof comprises:

1. a) a variable region of a heavy chain having the amino acid sequence of SEQ ID NO: 1 and a variable region of a light chain having the amino acid sequence of SEQ ID NO: 3;
2. b) a variable region of a heavy chain having the amino acid sequence of SEQ ID NO: 2 and a variable region of a light chain having the amino acid sequence of SEQ ID NO: 3;
3. c) variable region of a heavy chain having the amino acid sequence of SEQ ID NO: 2 and a variable region of a light chain having the amino acid sequence of SEQ ID NO: 4; or
4. d) a variable region of a heavy chain having the amino acid sequence of SEQ ID NO: 2 and a variable region of a light chain having the amino acid sequence of SEQ ID NO: 5.

[0012] The aforesaid antibody or the antigen binding fragment thereof, wherein the PD-1 is murine PD-1 which is mouse or rat PD-1.

[0013] The aforesaid antibody or antigen binding fragment thereof, wherein the antibody

1. a) binds to human PD-1 with a K_D of 2.15E-10 M or less; and
2. b) binds to mouse PD-1 with a K_D of 1.67E-08 M or less.

[0014] The aforesaid antibody, wherein the antibody

1. a) binds to human PD-1 with a K_D of 2.15E-10 M or less; and
2. b) binds to mouse PD-1 with a K_D of 1.67E-08 M or less, and

wherein the antibody exhibits at least one of the following properties:

1. a) binds to human PD-1 with a K_D of between 4.32E-10 M and 2.15E-10 M and to mouse PD-1 with a K_D of between 5.39E-8 M and 1.67E-8 M;
2. b) does not substantially bind to human CD28, CTLA-4;
3. c) increases T-cell proliferation;
4. d) increases interferon-gamma production; or
5. e) increases interleukin-2 secretion.

[0015] The present invention provides an antibody or an antigen binding fragment thereof, comprising:

1. a) a variable region of a heavy chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 1; and
2. b) a variable region of a light chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 3,

wherein the antibody specifically binds to PD-1;

1. a) a variable region of a heavy chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 2; and
2. b) a variable region of a light chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 3,

wherein the antibody specifically binds to PD-1;

1. a) a variable region of a heavy chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 2; and
2. b) a variable region of a light chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 4,

wherein the antibody specifically binds to PD-1;
or the antibody comprises:

1. a) a variable region of a heavy chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 2; and
2. b) a variable region of a light chain having an amino acid sequence selected from the group consisting of SEQ ID NO: 5,

wherein the antibody specifically binds to PD-1.

[0016] The sequence of the said antibody is shown in Table 1 and Sequence Listing. Antibodies other than 1H6, 2E5, 2G4, and 2C2 do not form part of the present invention.

Table 1 Sequence of the antibody

Clone ID	SEQ ID NO	Amino acid sequence	
1H6	Heavy chain	1	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAILGYFDYWGQG TMVTVSS
	Light chain	3	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLE IK
2E5	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	3	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGGTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLE IK
2G4	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	4	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGSTYL YWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGTD FTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLEI K
2C2	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	5	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGATYL YWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGTD FTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLEI K
A6W	Heavy chain	1	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAILGYFDYWGQG TMVTVSS
	Light chain	6	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGNTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLE IK
1G10	Heavy chain	1	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAILGYFDYWGQG TMVTVSS

Clone ID	SEQ ID NO		Amino acid sequence
	Light chain	5	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGATYL YWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGTD FTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLEI K
2B1	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	6	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGNTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHWPYTFGQGTKLE IK
L1I	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	7	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGNTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHAPYTFGQGTKLE
			DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGNTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHAPYTFGQGTKLE
5C4	Heavy chain	1	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAILGYFDYWGQG TMVTVSS
	Light chain	8	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGQTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHEPYTFGQGTKLEI K
8C10	Heavy chain	2	QVQLVQSGAEVKKPGSSVKVSCKASGFTFTTYYISW VRQAPGQGLEYLGYINMGSGGTNYNEKFKGRVTITA DKSTSTAYMELSSLRSEDTAVYYCAIIGYFDYWGQGT MVTVSS
	Light chain	9	DVVMTQSPLSLPVTLGQPASISCRSSQSLLDSDGQTY LYWFQQRPGQSPRRLIYLVSTLGSGVPDRFSGSGSGT DFTLKISRVEAEDVGVYYCMQLTHENYTFGQGTKLE IK

[0017] The antibody 1H6 which is an antibody according to the present invention comprises:

1. a) a heavy chain variable region CDR1 comprising SEQ ID NO: 10;
2. b) a heavy chain variable region CDR2 comprising SEQ ID NO: 11;
3. c) a heavy chain variable region CDR3 comprising SEQ ID NO: 12;
4. d) a light chain variable region CDR1 comprising SEQ ID NOs: 14;
5. e) a light chain variable region CDR2 comprising SEQ ID NOs: 19;
6. f) a light chain variable region CDR3 comprising SEQ ID NOs: 20;

wherein the antibody specifically binds to PD-1.

[0018] The antibody 2E5 which is another antibody according to the present invention comprises:

1. a) a heavy chain variable region CDR1 comprising SEQ ID NO: 10;
2. b) a heavy chain variable region CDR2 comprising SEQ ID NOs: 11;
3. c) a heavy chain variable region CDR3 comprising SEQ ID NOs: 13;
4. d) a light chain variable region CDR1 comprising SEQ ID NOs: 14;

5. e) a light chain variable region CDR2 comprising SEQ ID NO: 19;
6. f) a light chain variable region CDR3 comprising SEQ ID NO: 21;

wherein the antibody specifically binds to PD-1.

[0019] The antibody 2G4 which is another antibody according to the present invention comprises:

1. a) a heavy chain variable region CDR1 comprising SEQ ID NO: 10;
2. b) a heavy chain variable region CDR2 comprising SEQ ID NO: 11;
3. c) a heavy chain variable region CDR3 comprising SEQ ID NO: 13;
4. d) a light chain variable region CDR1 comprising SEQ ID NO: 15;
5. e) a light chain variable region CDR2 comprising SEQ ID NO: 19;
6. f) a light chain variable region CDR3 comprising SEQ ID NO: 21;

wherein the antibody specifically binds to PD-1.

[0020] The antibody 2C2 which is yet another antibody according to the present invention comprises:

1. a) a heavy chain variable region CDR1 comprising SEQ ID NO: 10;
2. b) a heavy chain variable region CDR2 comprising SEQ ID NO: 11;
3. c) a heavy chain variable region CDR3 comprising SEQ ID NO: 13;
4. d) a light chain variable region CDR1 comprising SEQ ID NO: 16;
5. e) a light chain variable region CDR2 comprising SEQ ID NO: 19;
6. f) a light chain variable region CDR3 comprising SEQ ID NO: 21;

wherein the antibody specifically binds to PD-1.

[0021] The CDR sequence of the said antibody is shown in Table 2 and Sequence Listing. Antibodies other than 1H6, 2E5, 2G4, and 2C2 are not part of the present invention.

Table 2 Sequence of the antibody

Clone ID.		SEQ ID NO	CDR1	SEQ ID NO	CDR2	SEQ ID NO	CDR3
1H6	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	12	LGYFDY
	Light chain	14	RSSQSLLDSDGGTYLY	19	LVSTLGS	20	MQLTHENYT
2E5	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	13	IGYFDY
	Light chain	14	RSSQSLLDSDGGTYLY	19	LVSTLGS	21	MQLTHWPYT
2G4	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	13	IGYFDY
	Light chain	15	RSSQSLLDSDGSTYLY	19	LVSTLGS	21	MQLTHWPYT
2C2	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	13	IGYFDY
	Light chain	16	RSSQSLLDSDGAIYLY	19	LVSTLGS	21	MQLTHWPYT
A6 W	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	12	LGYFDY
	Light chain	17	RSSQSLLDSDGNTYLY	19	LVSTLGS	21	MQLTHWPYT
1G1 0	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	12	LGYFDY
	Light chain	16	RSSQSLLDSDGAIYLY	19	LVSTLGS	21	MQLTHWPYT
2B1	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	13	IGYFDY
	Light chain	17	RSSQSLLDSDGNTYLY	19	LVSTLGS	21	MQLTHWPYT
L1I	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	13	IGYFDY
	Light	17	RSSQSLLDSDGNTYLY	19	LVSTLGS	22	MQLTHAPYT

Clone ID.		SEQ ID NO	CDR1	SEQ ID NO	CDR2	SEQ ID NO	CDR3
	chain						
5C4	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	12	LGYFDY
	Light chain	18	RSSQSLLDSDGQTYLY	19	LVSTLGS	23	MQLTHEPYT
8C1 0	Heavy chain	10	TYYIS	11	YINMGSGGTNYNEKFKG	12	LGYFDY
	Light chain	18	RSSQSLLDSDGQTYLY	19	LVSTLGS	20	MQLTHENYT

[0022] The antibodies of the invention can be humanized antibody.

[0023] The antibodies of the invention can exhibit at least one of the following properties:

1. a) binds to human PD-1 with a K_D of 2.15E-10 M or less and to mouse PD-1 with a K_D of 1.67E-08 M or less;
2. b) does not substantially bind to human CD28, CTLA-4;
3. c) increases T-cell proliferation;
4. d) increases interferon-gamma production; or
5. e) increases interleukin-2 secretion.

[0024] In a further aspect, the invention provides a nucleic acid molecule encoding the antibody, or antigen binding fragment thereof.

[0025] The invention provides a cloning or expression vector comprising the nucleic acid molecule encoding the antibody, or antigen binding fragment thereof.

[0026] The invention also provides a host cell comprising one or more cloning or expression vectors.

[0027] In yet another aspect, the invention provides a process, comprising culturing the host cell of the invention and isolating the antibody, wherein the antibody is prepared through immunization in SD rat with human PD-1 extracellular domain and mouse PD-1 extracellular domain.

[0028] The invention provides a transgenic mouse comprising human immunoglobulin heavy and light chain transgenes, wherein the mouse expresses the antibody of this invention.

[0029] The invention provides hybridoma prepared from the mouse of this invention, wherein the hybridoma produces said antibody.

[0030] In a further aspect, the invention provides pharmaceutical composition comprising the antibody, or the antigen binding fragment of said antibody in the invention, and one or more of a pharmaceutically acceptable excipient, diluent or carrier.

[0031] The invention provides an immunoconjugate comprising the said antibody, or antigen-binding fragment thereof in this invention, linked to a therapeutic agent.

[0032] Wherein, the invention provides a pharmaceutical composition comprising the said immunoconjugate and a pharmaceutically acceptable excipient, diluent or carrier.

[0033] Also disclosed but not forming part of the invention is a method for preparing an anti-PD-1 antibody or an antigen-binding fragment thereof comprising:

1. (a) providing: (i) a heavy chain variable region antibody sequence comprising a CDR1 sequence that is selected from a group consisting of SEQ ID NO: 10, a CDR2 sequence that is selected from a group consisting of SEQ ID NO: 11; and a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 12 and 13; and/or (ii) a light chain variable region antibody sequence comprising a CDR1 sequence that is selected from the group consisting of SEQ ID NOs: 14, 15, 16, 17 and 18, a CDR2 sequence that is selected from the group consisting of SEQ ID NO: 19, and a CDR3 sequence that is selected from the group consisting of SEQ ID NOs: 20, 21, 22 and 23; and
2. (b) expressing the altered antibody sequence as a protein.

[0034] The invention also provides the antibody according to the present invention for use in a method for the treatment or prophylaxis of an immune disorder or cancer.

[0035] In one embodiment, the method is a method for the treatment or prophylaxis of cancer by inhibiting growth of tumor cells in a subject, said method comprising administering to the subject a therapeutically effective amount of the said antibody to inhibit growth of the tumor cells.

[0036] In one embodiment, the method is a method, wherein the tumor cells are of a cancer selected from a group consisting of melanoma, renal cancer, prostate cancer, breast cancer, colon cancer, lung cancer, bone cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular malignant melanoma, uterine cancer, ovarian cancer, and rectal cancer.

[0037] In one embodiment, the method is a method, wherein the antibody is a humanized antibody.

The features and advantages of this invention

[0038] The inventors have generated a humanized antibody against PD-1 utilizing the proprietary hybridoma technology. The antibodies reported in this invention have high binding affinity, specifically binding to both human and mouse PD-1 protein without cross-family reactions; and potent modulating immune responses, including enhancing T cell proliferation and increasing cytokine IFN-γ and interleukin-2 production.

[0039] New anti-PD-1 antibodies binding to mouse PD-1 are derived from immuned rats, which overcomes the disadvantage that is anti-PD-1 antibodies can not be used in pre-clinical mouse model; and the humanized level is close to 100% after sequence humanization, greatly reducing the adverse effects of drugs used in the human body.

Brief Description of the Drawings

[0040] Antibodies other than 1H6, 2E5, 2G4, and 2C2 do not form part of the present invention.

Figure 1 shows graphs of hybridoma antibodies binding to cell surface human and mouse PD-1. Figure. 1A shows binding to human PD-1. Figure 1B shows binding to mouse PD-1.

Figure 2 shows the result from first around mutagenesis library screen. Sequence and analysis mutation on high affinity clones for the second around mutation.

Figure 3 shows the results of cross-species test by FACS. Figure 3A shows binding to human PD-1 transfected CHO-S cells. Figure 3B shows binding to mouse PD-1 transfected 293F cells. Figure 3C shows binding to activated cynomolgus PBMC. Note: the isotype was human IgG4 kappa. The same below.

Figure 4 shows the result of cross-species test by ELISA. Figure 4A shows binding to human PD-1. Figure 4B shows binding to mouse PD-1. Figure 4C shows binding to cynomolgus PD-1.

Figure 5 shows the result of cross-family test. The anti-PD-1 antibodies bind specifically to human PD-1, but not to CD28 and CTLA-4.

Figure 6A shows the result of anti-PD-1 antibodies blocking human PD-L1 binding to PD-1 transfected CHO-S cells. Figure 6B shows the result of anti-PD-1 antibodies blocking mouse PD-L1 binding to PD-1 transfected 293F cells.

Figure 7 shows that the anti-PD-1 antibodies could block human PD-L2 binding to PD-1.

Figure 8A-8B show the results of epitope binning assay suggesting that the anti-PD-1 antibodies are in the same or close epitope bin as benchmark antibodies. Figure 8A shows binning against WBP305BMK1 (US9084776). Figure 8B shows binning against Keytruda (US8168757).

Figure 9 shows the cross-reactivity of anti-PD-1 antibodies with human/mouse PD-1. 2 μg/mL of each antibody were coated at 96-well plate overnight and incubated with hPD-1/mPD-1-His protein, then HRP-anti-His antibody were added for detection.

Figure 10 shows the Hot spot residues mapped on hPD-1 structure. (A). hPD-L1 binding site. Data were obtained from the literature Zak et al. 2015. (B-C). Binding site of antibody W3052_r16.88.9 and Keytruda, respectively. Data were from table 8. Colors on the pictures are to help distinguish the differences between epitopes.

Figure 11 shows comparison between human and murine PD-1. Their obvious structural differences (BC loop and C'D loop (or C" strand on mPD-1)) were marked in orange color. (A). Structures of hPD-1 (PDB code 4ZQK). The missing loop (Asp85-Asp92) were remolded based on its NMR structures (PDB code 2M2D). (B). Structure of mPD-1 (PDB code 3BIK).

Figure 12A-12C show the results of human allo-MLR demonstrating the anti-PD-1 antibodies can enhance the function of human $CD4^+$

T cell. Figure 12A shows anti-PD-1 antibodies increase IL-2 secretion in a dose-dependent manner. Figure 12B shows anti-PD-1 antibodies increase IFN-γ secretion in a dose-dependent manner. Figure 12C shows anti-PD-1 antibodies increase $CD4^+$ T cells proliferation in a dose-dependent manner.

Figure 13A-13C show the results of mouse allo-MLR demonstrating that the anti-PD-1 antibodies can enhance the function of mouse $CD4^+$ T cell. Figure 13A shows anti-PD-1 antibodies increase IL-2 secretion in a dose-dependent manner. Figure. 13B shows anti-PD-1 antibodies increase IFN-γ secretion in a dose-dependent manner. Figure 13C shows anti-PD-1 antibodies increase $CD4^+$ T cells proliferation in a dose-dependent manner.

Figure 14A-14B show the results of human allo-MLR demonstrating the anti-PD-1 antibodies can enhance the function of human $CD4^+$ T cell. Figure 14A shows anti-PD-1 antibodies increase IFN-γ secretion in a dose-dependent manner. Figure 14B shows anti-PD-1 antibodies increase $CD4^+$ T cells proliferation in a dose-dependent manner.

Figure 15 demonstrates that the anti-PD-1 antibodies can reverse the suppressive function of Tregs. Figure 15A shows anti-PD-1 antibodies can restore the IFN-γ secretion. Figure 15B shows anti-PD-1 antibodies can restore the T-cell proliferation.

Figure 16 shows the result of ADCC test demonstrating the anti-PD-1 antibodies do not mediate ADCC activity on activated $CD4^+$ T cells.

Figure 17 shows the result of CDC test demonstrating the anti-PD-1 antibodies do not mediate CDC activity on activated $CD4^+$ T cells.

Figure 18 shows body weight changes in syngeneic tumor nude mice model after treatment of 2E5. The data point represents the average body weight; error bars represent the standard error (SEM).

Figure 19 shows relative weight changes (%). Relative change in body weight was calculated based on body weight at the start of the administration. The data point represents the average body weight; error bars represent the standard error (SEM).

Figure 20 shows tumor growth curve in CloudmanS91 syngeneic tumor nude mice model after treatment of 2E5. The data point represents the average body weight; error bars represent the standard error (SEM).

Figure 21 shows survival curve in CloudmanS91 syngeneic tumor nude mice model after treatment of 2E5.

Detailed description

[0041] In order that the present invention may be more readily understood, certain terms are first defined. Additional definitions are set forth throughout the detailed description.

[0042] The terms "Programmed Death 1", "Programmed Cell Death 1", "Protein PD-1", "PD-1", "PD1", "PDCD1", "hPD-1" and "hPD-F" are used interchangeably, and include variants, isoforms, species homologs of human PD-1, and analogs having at least one common epitope with PD-1.

[0043] The term "antibody" as referred to herein includes whole antibodies and any antigen- binding fragment (i.e., "antigen-binding portion") or single chains thereof. An "antibody" refers to a protein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds, or an antigen-binding portion thereof. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as VH) and a heavy chain constant region. The heavy chain constant region is comprised of three domains, CH1, CH2 and CH3. Each light chain is comprised of a light chain variable region (abbreviated herein as VL) and a light chain constant region. The light chain constant region is comprised of one domain, CL. The VH and VL regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions (FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen.

[0044] The term "antibody," as used in this disclosure, refers to an immunoglobulin or a fragment or a derivative thereof, and encompasses any polypeptide comprising an antigen-binding site, regardless whether it is produced in vitro or in vivo. The term includes, but is not limited to, polyclonal, monoclonal, monospecific, polyspecific, non-specific, humanized, single-chain, chimeric, synthetic, recombinant, hybrid, mutated, and grafted antibodies. The term "antibody" also includes antibody fragments such as Fab, F(ab')2, Fv, scFv, Fd, dAb, and other antibody fragments that retain antigen-binding function, i.e., the ability to bind PD-1 specifically. Typically, such fragments would comprise an antigen-binding fragment.

[0045] The terms "antigen-binding fragment," "antigen-binding domain," and "binding fragment" refer to a part of an antibody molecule that comprises amino acids responsible for the specific binding between the antibody and the antigen. In instances, where an antigen is

large, the antigen-binding fragment may only bind to a part of the antigen. A portion of the antigen molecule that is responsible for specific interactions with the antigen-binding fragment is referred to as "epitope" or "antigenic determinant."

[0046] An antigen-binding fragment typically comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH), however, it does not necessarily have to comprise both. For example, a so-called Fd antibody fragment consists only of a VH domain, but still retains some antigen-binding function of the intact antibody.

[0047] In line with the above the term "epitope" defines an antigenic determinant, which is specifically bound/identified by a binding fragment as defined above. The binding fragment may specifically bind to/interact with conformational or continuous epitopes, which are unique for the target structure, e.g. the human and murine PD-1. A conformational or discontinuous epitope is characterized for polypeptide antigens by the presence of two or more discrete amino acid residues which are separated in the primary sequence, but come together on the surface of the molecule when the polypeptide folds into the native protein/antigen. The two or more discrete amino acid residues contributing to the epitope are present on separate sections of one or more polypeptide chain(s). These residues come together on the surface of the molecule when the polypeptide chain(s) fold(s) into a three-dimensional structure to constitute the epitope. In contrast, a continuous or linear epitope consists of two or more discrete amino acid residues, which are present in a single linear segment of a polypeptide chain.

[0048] The term "binds to an epitope of PD-1" refers to the antibodies have specific binding for a particular epitope of PD-1, which may be defined by a linear amino acid sequence, or by a tertiary, i.e., three-dimensional, conformation on part of the PD-1 polypeptide. Binding means that the antibodies affinity for the portion of PD-1 is substantially greater than their affinity for other related polypeptides. The term "substantially greater affinity" means that there is a measurable increase in the affinity for the portion of PD-1 as compared with the affinity for other related polypeptides. Preferably, the affinity is at least 1.5-fold, 2-fold, 5-fold 10-fold, 100-fold, 10^3-fold, 10^4-fold, 10^5-fold, 10^6-fold or greater for the particular portion of PD-1 than for other proteins. Preferably, the binding affinity is determined by enzyme-linked immunoabsorbent assay (ELISA), or by fluorescence-activated cell sorting (FACS) analysis or surface Plasmon resonance (SPR). More preferably, the binding specificity is obtained by fluorescence-activated cell sorting (FACS) analysis.

[0049] The term "cross-reactivity" refers to binding of an antigen fragment described herein to the same target molecule in human and murine (mouse or rat). Thus, "cross-reactivity" is to be understood as an interspecies reactivity to the same molecule X expressed in different species, but not to a molecule other than X. Cross-species specificity of a monoclonal antibody recognizing e.g. human PD-1, to a murine (mouse or rat) PD-1, can be determined, for instance, by FACS analysis.

[0050] As used herein, the term "subject" includes any human or nonhuman animal. The term "nonhuman animal" includes all vertebrates, e.g., mammals and non-mammals, such as nonhuman primates, sheep, dogs, cats, horses, cows, chickens, amphibians, reptiles, etc. Except when noted, the terms "patient" or "subject" are used interchangeably.

[0051] The terms "treatment" and "therapeutic method" refer to both therapeutic treatment and prophylactic/preventative measures. Those in need of treatment may include individuals already having a particular medical disorder as well as those who may ultimately acquire the disorder.

Examples

[0052] Antibodies other than 1H6, 2E5, 2G4, and 2C2 do not form part of the present invention.

Example 1: Research materials preparation

1. Immunogen generation

[0053] DNAs encoding the ECD or full length of PD-1 and PD-L1 were synthesized and inserted into the expression vector pcDNA3.3. Max-prep the plasmid DNAs and the inserted DNA sequences were verified by sequencing. Fusion proteins PD-1 ECD and PD-L1 ECD containing various tags, including human Fc, mouse Fc and His tags, were obtained by transfection of human PD-1 ECD gene into CHO-S or HEK293 cells. After 5 days, supernatants were harvested from the culture of transient transfected cells. The fusion proteins were purified and quantified for usage of immunization and screening.

2. Stable cell lines establishment

[0054] In order to obtain tools for antibody screening and validation, we generated PD-1 and PD-L1 transfecting cell lines. Briefly, CHO-K1 or 293F cells were transfected with pcDNA3.3 expression vector containing full-length PD-1 or PD-L1 using Lipofectamine 2000

Transfection kit according to manufacturer's protocol. 48-72 hours post transfection; the transfected cells were cultured in medium containing Blasticidin or G418 to select the cells that had PD-1 or PD-L1 genes stably incorporated into their genomic DNAs. Meanwhile the cells were checked for interested genes PD-1 and PD-L1 expression. Once the expression verified, single clones of interested were picked by limited dilution and scaled up to large volumes. The established monoclonal cell lines were then maintained in medium containing lower dose of antibiotics Blasticidin or G418.

Example 2: Antibody hybridoma generation

1. Immunization

[0055] Female SD rats, at 6-8 weeks of age, were immunized with 10 µg/animal of human PD-1 ECD protein and 10 µg/animal of mouse PD-1 ECD protein in TiterMax by footpad injection for prime, and were boosted twice a week with human PD-1 ECD protein or mouse PD-1 ECD protein in Aluminium alternately. The serum antibody titers were measured by ELISA or FACS every two weeks.

2. Cell fusion

[0056] When the serum antibody titer was sufficiently high, rats were given a final boost with both human and mouse PD-1 ECD protein in the equal volume of D-PBS (Dulbecco's Phosphate Buffered Saline) without adjuvant. The cell fusion was performed as follows: preparing myeloma cells SP2/0, myeloma cells were thawed the week before the fusion, and were split at 1:2 each day until the day before the fusion to keep in logarithmic growth. B lymphocytes isolated from lymph node of immunized SD rats were combined with myeloma cells (at 1:1 ratio). The cells were treated with Trypsin and the reaction was stopped by FBS. Cell mixture was then washed and re-suspended in ECF solution at 2×10^6 cells/ml for ECF. After electronic cell fusion (BTX2000), cell suspension from the fusion chamber was immediately transferred into a sterile tube containing more medium, and incubated for at least 24 hours in a 37 °C incubator. The cell suspension was then mixed and transferred into 96-well plates (1×10^4 cells/well). The 96-well plates were cultured at 37 °C, 5% CO_2, and were monitored periodically. When the clones were big enough (after 7-14 days), 100 µL of supernatant were transferred from the tissue culture plates to 96-well assay plates for antibody screening.

3. First, second and confirmation screen of hybridoma supernatants

[0057] ELISA assay was used as first screen method to test the binding of hybridoma supernatants to human or mouse PD-1 protein. Briefly, plates (Nunc) were coated with human or mouse PD-1 ECD at 1 µg/ml overnight at 4 °C. After blocking and washing, the hybridoma supernatants were loaded to the coated plates and incubated at room temperature for 1 h. The plates were then washed and subsequently incubated with secondary antibody goat anti rat IgG Fc HRP (Bethyl) for 1 h. After washing, TMB substrate was added and the reaction was stopped by 2M HCl. The absorbance at 450 nm was read using a microplate reader (Molecular Device).

[0058] In order to confirm the native binding of anti-PD-1 antibodies on conformational PD-1 molecules expressed on cell membrane, FACS analysis was performed using PD-1 transfected cell lines as second screening. CHO-S cells expressing human PD-1 or 293F cells expressing mouse PD-1 were transferred into 96-well U-bottom plates (Corning) at a density of 1×10^5 cells/well. The hybridoma supernatants were then added and incubated with the cells for 1 h at 4 °C. After washing with 1xPBS/1%BSA, the secondary antibody goat anti rat FITC (Jackson ImmunoResearch Lab) was applied and incubated with cells at 4 °C in the dark for 1 h. The cells were then washed and resuspended in 1×PBS/1%BSA or fixed with 4% paraformldehyde, and analyzed by flow cytometery (BD) and FlowJo software. Antibody binding to parental CHO-S or 293F cell line was used as negative control, respectively.

[0059] To select potential antagonistic hits, selected antibodies were tested for their ability to block the binding of the ligand PD-L1 to PD-1 transfected cells by FACS analysis. CHO-S cells expressing human PD-1 or 293F cells expressing mouse PD-1 were transferred into 96-well U-bottom plates (BD) at a density of 1×10^5 cells/well. Hybridoma supernatants were added and incubated with the cells at 4 °C for 1 h. After washing, mouse Fc fusion-human PD-L1 protein or mouse Fc fusion-mouse PD-L1 protein was added and incubated at 4 °C for 1 h. The secondary antibody goat anti mouse IgG Fc FITC antibody (no cross-reactivity to rat IgG Fc, Jackson ImmunoResearch Lab) was incubated with cells at 4 °C in the dark for 1 h. The cells were then washed and resuspended in 1×PBS/1%BSA or fixed with 4% paraformldehyde, and analyzed by flow cytometery (BD) and FlowJo software.

[0060] Figure. 1 shows graphs of 16 hybridoma antibodies binding to cell surface human and mouse PD-1. Figure. 1A shows binding to human PD-1. Figure. 1B shows binding to mouse PD-1.

4. Hybridoma subcloning

[0061] Once specific binding and blocking activity were verified through first and confirmation screening, the positive hybridoma cell lines were used for subcloning. Briefly, for each hybridoma cell line, cells were counted and diluted to give 5 cells, 1 cell or 0.5 cell per 200 µL cloning medium. The cell suspension was plated 200 µL/well into 96-well plates, one plate at 5 cells/well, one plate at 1 cell/well and four plates at 0.5 cell/well. Plates were cultured at 37 °C, 5% CO_2, till they were ready to be screened by binding ELISA or FACS as described above. The ESN of selected single clones were collected, and the antibodies were purified for further characterization.

5. Subtypes Testing

[0062] 50 µL of goat anti-rat IgG1, IgG2a, IgG2b, IgG2c, IgG or IgM antibodies (1 µg/mL) were coated in microtiter plates (Nunc) per well overnight. After blocking, 50 µL of hybridoma supernatant samples were added to each well, incubated for 2 hours at room temperature. Goat anti-rat IgG kappa or HRP labeled lambda light chain secondary antibody (Bethyl) is a detection antibody. Using TMB substrate for color, the reaction was then quenched with 2 M HCl. The value of absorbs light at 450 nm is read using a microplate reader (Molecular Device).

[0063] Table 3 shows the subtype results of 16 hybridoma antibodies. 7 antibodies are polyclonal antibodies, and 9 antibodies are IgG2a kappa subtype. Considering the needs of anti-PD-1 antibody to avoid the role of ADCC and CDC in *vivo,* the humanized antibody will be built as human IgG4 kappa subtype.

Table 3. Subtypes of the hybridoma antibodies

Number	antibody	kappa			
		IgG1	IgG2a	IgG2b	IgM
1	W3052_r16.6.25	-	-	+	weak
2	W3052_r16.14.6	+	+	-	-
3	W3052_r16.14.16	-	+	-	+
4	W3052_r16.16.14	+	+	-	+
5	W3052_r16.53.26	+	+	-	-
6	W3052_r16.68.22	-	+	-	-
7	W3052_r16.68.41	-	+	-	-
8	W3052_r16.81.3	-	-	+	+
9	W3052_r16.88.9	-	+	-	-
10	W3052_r16.88.21	weak	+	-	-
11	W3052_r16.88.29	-	weak	-	-
12	W3052_r16.88.32	-	+	-	-
13	W3052_r16.114.2		+	-	-
14	W3052_r16.114.8	-	+	-	-
15	W3052_r16.114.15	-	+	-	-
16	W3052_r16.114.39	-	+	-	-

Example 3: Antibody hybridoma cell sequence and humanized antibody molecules construction and affinity maturation

1. Antibody hybridoma cell sequence

[0064] RNAs were isolated from monoclonal hybridoma cells with Trizol reagent. The VH and VL of PD-1 chimeric antibodies were amplified as follows: RNA is first reverse transcribed into cDNA using a reverse transcriptase as described here,

Reaction system (20 µL)

[0065]

10×RT Buffer	2.0 µL
25×dNTP Mix (100 mM)	0.8 µL
10×RT Random Primers/oligodT/specific primer	2.0 µL
MultiScribe™ Reverse Transcriptase	1.0 µL

RNase Inhibitor	1.0 μL
RNA	2 μg
Nuclease-free H_2O	to 20.0 μL

Reaction condition

[0066]

	Step 1	**Step 2**	**Step3**	**Step4**
Temperature (°C)	25	37	85	4
Time	10 min	120 min	5	∞

[0067] The resulting cDNA was used as templates for subsequent PCR amplification using primers specific for interested genes. The PCR reaction was done as follows:

cDNA	1 μL
Ex PCR buffer	5 μL
dNTP	2 μL
ExTaq	0.5 μL
P1 (25 pM)	0.5 μL
P2 (25 pM)	0.5 μL
ddH_2O	40.5 μL

Reaction condition:

[0068]

94 °C	3 min	
94 °C	30s	} 30 cycles
60 °C	30s	
72 °C	1 min	
72 °C	10 min	

[0069] The resulting PCR product (10 μL) was ligated with pMD18-T vector. Top 10 competent cells were transformed with 10 μL of the ligation product. Positive clones were checked by PCR using M13-48 and M13-47 primers followed by sequencing.

2. Humanized antibody molecule construction

[0070] The rat anti-PD-1 antibody from hybridomas were selected and humanized according to the high affinity and specificity of anti-PD-1 antibody binding to PD-1, improving the homology with human antibody sequence. The said humanized usage is called as CDR-grafting technique. The variable region gene of antibody such as FR regions and CDR regions were divided by KABAT system and IMGT system. In antibody database, based on the alignments of binding sequence homology and structural similarity, the gene of murine region FR1-3 was replaced by humanized variable region FR1-3, region FR4 of the murine gene was replaced by humanized FR4 region derived from JH and JK genes which had the most similar structures. After verifying the template sequence and codon optimization, the heavy chain variable region and light chain variable region were synthesized and cloned into the expression vector, and then expressing the humanized antibody.

[0071] According to the binding ability to cell surface human and mouse PD-1, W3052_r16.88.9 and W3052_r16.81.3 was selected for humanization. Table.2 shows the analysis of humanization scores. The clones W3052-16.88-z9-IgG4 (42720) was selected for affinity maturation considering all these factors such as better affinity and humanization scores (Table.4).

Table.4

Lead antibody		FR1		FR2	FR3	FR4		Humanization score
W3052_r16.88.hAb1 40798	WBP305_r16.88-hVH 1-m	IGHV1-6 9*06	100%	100%	93.30%	IGHJ3*01	100%	99.16%
	WBP305_r16.88-VL1	IGKV2-2 9*02	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.88.hAb2 40764	WBP305_r16.88-hVH 2	IGHV1-6 9*06	100%	(IGHV 1-8*01) 100%	93.30%	IGHJ3*01	100%	99.16%
	WBP305_r16.88-VL1	IGKV2-2 9*02	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.88.hAb3 40766	WBP305_r16.88-hVH 3	IGHV1-6 9*06	100%	85.70 %	93.30%	IGHJ3*01	100%	97.38%
	WBP305_r16.88-VL1	IGKV2-2 9*02	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.88.hAb4 40770	WBP305_r16.88-hVH 1	IGHV1-6 9*06	100%	100%	93.30%	IGHJ3*01	100%	99.16%
	WBP305_r16.88-VL2	IGKV2-3 0*01	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.88.hAb5 40773	WBP305_r16.88-hVH 2-m	IGHV1-6 9*06	100%	(IGHV 1-8*01) 100%	93.30%	IGHJ3*01	100%	99.16%
	WBP305_r16.88-VL2	IGKV2-3 0*01	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.88.hAb6 40800	WBP305_r16.88-hVH 3-m	IGHV1-6 9*06	100%	85.70 %	93.30%	IGHJ3*01	100%	97.38%
	WBP305_r16.88-VL2	IGKV2-3 0*01	100%	100%	100%	IGKJ2*01	100%	
W3052_r16. 88-z7-lgG4 42691	WBP305_r16.88-hVH 1	IGHV1-6 9*06	100%	100%	100%	IGHJ3*01	100%	100%
	WBP305_r16.88-VL1	IGKV2-2 9*02	100%	100%	100%	IGKJ2*01	100%	
W3052_r16. 88-z8-lgG4 42715	WBP305_r16.88-hVH 2	IGHV1-6 9*06	100%	(IGHV 1-8*01) 100%	100%	IGHJ3*01	100%	100%
	WBP305_r16.88-VL2	IGKV2-3 0*01	100%	100%	100%	IGKJ2*01	100%	
W3052_r16. 88-z9-lgG4 42720	WBP305_r16.88-hVH 3	IGHV1-6 9*06	100%	85.70 %	100%	IGHJ3*01	100%	98.20%
	WBP305_r16.88-VL2	IGKV2-3 0*01	100%	100%	100%	IGKJ2*01	100%	
W3052_r16.81.hAb1 40779	WBP305_r16.81-VH1	IGHV3-7 *03	100%	100%	100%	IGHJ1*01	100%	100%
	WBP305_r16.81-VL1	IGKV1D-16*02	100%	100%	100%	IGKJ4*01	100%	
W3052_r16.81.hAb2 40781	WBP305_r16.81-VH2	IGHV3-9 *01	100%	100%	100%	IGHJ1*01	100%	100%
	WBP305_r16.81-VL1	IGKV1D-16*02	100%	100%	100%	IGKJ4*01	100%	
W3052_r16.81.hAb3 40784	WBP305_r16.81-VH1	IGHV3-7 *03	100%	100%	100%	IGHJ1*01	100%	100%
	WBP305_r16.81-VL2	IGKV1-3 9*01	100%	100%	100%	IGKJ4*01	100%	
W3052_r16.81.hAb4 40787	WBP305_r16.81-VH2	IGHV3-9 *01	100%	100%	100%	IGHJ1*01	100%	100%
	WBP305_r16.81-VL2	IGKV1-3 9*01	100%	100%	100%	IGKJ4*01	100%	

3. Affinity maturation

[0072] Each amino acid of three complementary-determining regions (VH CDR3, VK CDR1, and VK CDR3) of parental clone was individually mutated to other 20 amino acids using a hybridization mutagenesis method. DNA primers containing a NNS codon encoding twenty amino acids were used to introduce mutation to each targeted CDR position. The individual degenerate primers were used in hybridization mutagenesis reactions. Briefly, each degenerate primer was phosphorylated, and then used in a 10:1 ratio with uridinylated ssDNA. The mixture was heated to 85 °C for 5 minutes then cooled down to 55 °C over 1 hour. Thereafter, T4 ligase and T4 DNA polymerase were added and mix was incubated for 1.5 hours at 37 °C. Synthesis products for VH and VL CDRs were pooled respectively. Typically, 200 ng of the pooled library DNA was electroporated into BL21 for plaque formation on BL21 bacterial lawn or for production of scFv fragments.

[0073] The primary screen consisted of a single point ELISA (SPE) assay which was carried out using periplasmic extract (PE) of bacteria grown in 96-well plates (deep well). Briefly, this capture ELISA involved coating individual wells of a 96-well Maxisorp Immunoplate with anti-c-myc antibody in coating buffer (200 mM Na_2CO_3/$NaHCO_3$) at pH 9.2 overnight at 4 °C. The next day, the plate was blocked with Casein for 1 h at room temperature. scFv PE was then added to the plate and incubated at room temperature for 1 hr. After washing, biotinylated antigen protein was added to the well and the mixture was incubated for 1h at room temperature. This was followed by incubation with Streptavidin-HRP conjugate for 1h at room temperature. HRP activity was detected with TMB substrate and the reaction was quenched with 2 M HCl. Plates were read at 450 nm. Clones exhibiting an optical density (OD) signal at 450 nm greater than the parental clone were picked and re-assayed by ELISA (as described above) in duplicate to confirm positive results. Clones that repeatedly exhibited a signal greater than that of the parental antibody were sequenced. The scFv protein concentration of each clone that had a CDR change was then determined by a quantitative scFv ELISA, where a scFv with known concentration was used as a reference. The scFv protein concentration was determined by comparing the ELISA signals with signals generated by the reference scFv. The binding assay was repeated once more for all positive variants under normalized scFv concentration in order to determine the relative binding affinity of the mutant scFv and the parental antibody.

[0074] The point mutations in VH and VL determined to be beneficial for binding to antigen were further combined to gain additional binding synergy. The combinatorial mutants were expressed as scFv and screened using the capture ELISA. Clones exhibiting an OD signal at 450 nm greater than the parental clone were sequenced and further confirmed by binding ELISA as described above.

[0075] After affinity maturation, a total of 10 humanized antibodies (2E5, 2G4, 1G10, 2C2, 2B1, 8C10, 1H6, 5C4, A6W and L1I) were obtained. Figure 2 showed the result from first round mutagenesis library screen. Sequence and affinity data of 10 humanized antibodies in human, cynomolgus monkeys and mice were shown in Table 5.

[0076] Table.5 showed the result from second round mutagenesis library screen. The clones 1H6, 2E5, 2G4 and 2C2 were selected for further analysis.

Table 5

Name	VHCDR3	VKCDR1	VKCDR3	Bmax(Human)	Kd (Human)	Bmax (Mouse)	Kd (Mouse)	Bmax (Cynomolgus)	Kd (Cynomolgus)
2E5	I	LDSDGGTYLYW (SEQ ID NO:25)	MQLTHWPYTFGQ (SEQ ID NO:30)	3.279	0.0675	0.4696	0.0443	1.72	0.1088
2G4	I	LDSDGSTYLYW (SEQ ID NO:26)	MQLTHWPYTFGQ (SEQ ID NO:30)	3.371	0.0708	0.4793	0.0426	1.718	0.1057
1G10	L	LDSDGATYLYW (SEQ ID NO:27)	MQLTHWPYTFGQ (SEQ ID NO:30)	2.600	0.0711	0.2997	0.0718	1.082	0.1224
2C2	I	LDSDGATYLYW (SEQ ID NO:27)	MQLTHWPYTFGQ (SEQ ID NO:30)	3.175	0.082	0.416	0.049	1.668	0.116
2B1	I	LDSDGNTYLYW (SEQ ID NO:28)	MQLTHWPYTFGQ (SEQ ID NO:30)	3.019	0.0912	0.3393	0.0346	1.207	0.1142
8C10	I	LDSDGQTYLYW (SEQ ID NO:29)	MQLTHENYTFGQ (SEQ ID NO:31)	2.307	0.104	0.437	0.038	1.109	0.280
1H6	L	LDSDGGTYLYW (SEQ ID NO:25)	MQLTHWPYTFGQ (SEQ ID NO:30)	3.348	0.1114	0.2213	0.0466	0.3171	0.0977
5C4	L	LDSDGQTYLYW (SEQ ID NO:29)	MQLTHEPYTFGQ (SEQ ID NO:32)	2.649	0.236	0.164	0.027	0.258	0.292
A6W	L	LDSDGNTYLYW (SEQ ID NO:28)	MQLTHWPYTFGQ (SEQ ID NO:30)	2.571	0.2885	0.1736	0.0294	0.0787	0.0016
L1I	I	LDSDGNTYLYW (SEQ ID NO:28)	MQLTHAPYTFGQ (SEQ ID NO:33)	1.048	1.8370	0.1048	0.0050	0.05175	N/A

4.Antibody Purification

[0077] The vector containing affinity matured humanized antibody were transfected into 293F cells for antibody production and expression. Antibodies in the supernatant of 293F cells were purified using Protein A affinity chromatography.

Example 4: Characterization of humanized antibody

1. Cross-reactivity to human, cynomolgus and mouse PD-1 (cross-species)

1.1 FACS

[0078] Cross-reactivity was measured by FACS and ELISA. For FACS, the anti-PD-1 antibodies were tested binding to cell surface human, mouse and cynomolgus PD-1 as described in Example 2.3.

[0079] Figure 3 showed the results of cross-species test by FACS. Figure 3A showed binding to human PD-1 transfected CHO-S cells. The antibodies can bind specifically to the human PD-1 with EC50 of 2.20-2.78 nM. Figure.3B showed binding to mouse PD-1 transfected 293F cells. The antibodies can bind specifically to the mouse PD-1 with EC50 of 11.8-15.1 nM. Figure.3C showed binding to activated cynomolgus PBMC in a dose dependent way. The isotype was human IgG4 kappa. The same below.

1.2 Cross-reactivity to human, cynomolgus and mouse PD-1 (cross-species)

[0080] For ELISA, plates (Nunc) were coated with human, cynomolgus or mouse PD-1 (Sino Biological) at 1 μg/ml overnight at 4 °C. After blocking and washing, antibodies were serially diluted in blocking buffer and added to the plates and incubated at room temperature for 1 h. The plates were then washed and subsequently incubated with secondary antibody goat anti human IgG HRP (Bethyl) for 1 h. After washing, TMB substrate was added and the reaction was stopped by 2 M HCl. The absorbance at 450 nm was read using a microplate reader (Molecular Device).

[0081] Figure 4 showed the result of cross-species test by ELISA. Figure 4A showed binding to human PD-1. Figure 4B showed binding to mouse PD-1. Figure 4C showed binding to cynomolgus PD-1.

2. Cross-reactivity to human PD-1 family members CD28, CTLA4

[0082] Constructed cell lines that respectively express human PD-1, CD28, CTLA-4 or ICOS were transferred in to 96-well U-bottom plates (BD) at a density of 2×10^5 cells/well. Testing antibodies were diluted in wash buffer (1×PBS/1%BSA) and incubated with cells at 4 °C for 1 h. After washing, the secondary antibody goat anti-human IgG Fc FITC (Jackson ImmunoResearch Lab) was added and incubated at 4 °C in the dark for 1 h. The cells were then washed once and resuspended in 1×PBS/1%BSA, and analyzed by flow cytometery (BD) and FlowJo software.

[0083] Figure 5 showed the result of cross-family test. The anti-PD-1 antibodies can bind specifically to human PD-1, but not to CD28 and CTLA-4.

3. Blocking of ligand binding to PD-1

[0084] 3.1 The ability of anti-PD-1 antibodies to block PD-L1 binding to PD-1 was tested by FACS as described in Example 2.3.

[0085] 3.2 The ability of anti-PD-1 antibodies to block PD-L2 binding to PD-1 was tested by ELISA. Briefly, plates (Nunc) were coated with human PD-1 at 1 μg/ml overnight at 4 °C. Antibodies were serially diluted in blocking buffer and mixed with his tag conjugated PD-L2. After blocking and washing the coated plates, the antibody/PD-L2 mixture were added to the plates, then incubated at room temperature for 1 h. The plates were then washed and subsequently incubated with secondary antibody goat anti-his HRP (GenScript) for 1 h. After washing, TMB substrate was added and the reaction was stopped by 2 M HCl. The absorbance at 450 nm was read using a microplate reader (Molecular Device).

[0086] Figure 6A showed the result of anti-PD-1 antibodies blocking human PD-L1 binding to PD-1 transfected CHO-S cells. Figure 6B

shows the result of anti-PD-1 antibodies blocking mouse PD-L1 binding to PD-1 transfected 293F cells. Figure 7 showed that the anti-PD-1 antibodies could block human PD-L2 binding to PD-1 in a dose-dependent manner.

4. Full kinetic binding affinity tested by surface Plasmon resonance (SPR)

[0087] Antibodies were characterized for affinity and binding kinetics to PD-1 by SPR assay using ProteOn XPR36 (Bio-Rad). Protein A protein (Sigma) was immobilized to a GLM sensor chip (Bio-Rad) through amine coupling. Purified antibodies were flowed over the sensor chip and captured by the Protein A. The chip was rotated 90° and washed with running buffer (1×PBS/0.01% Tween20, Bio-Rad) until the baseline was stable. Seven concentrations of human PD-1 and running buffer were flowed through the sensor chip at a flow rate of 30 μL/min for an association phase of 180 s, followed by 300s dissociation. After regeneration, seven concentration of mouse PD-1 and running buffer were flowed through the sensor chip at a flow rate of 30 μL/min for an association phase of 180 s, followed by 300 s dissociation. The chip was regenerated with pH 1.5 H_3PO_4 after each run. The association and dissociation curve was fit by 1:1 Langmuir binding model using ProteOn software.

[0088] Table.6A-6B showed the results of full kinetic binding affinity to human and mouse PD-1 by SPR. WBP305BMK1 was synthesized according to the clone of 5C4 from BMS patent US9084776B2. Keytruda was the anti-PD-1 drug from Merck. The same below. The results showed that the affinity ability to human PD-1 by SPR assay was from 1.43E-8 to 5.64E-9 mol/L. Comparing WBP305BMK1 with Keytruda, the K_D value of antibody 2E5, 2G4 or 2C2 was much smaller, illustrating that 2E5, 2G4 or 2C2 had better binding ability to human PD-1. In addition, the affinity ability to mouse PD-1 was from 9.37E-9 to 3.89E-9 mol/L.

Table 6A

Analyte	Ligand	ka (1/Ms)	kd (1/s)	KD (M)	Chi^2 (RU^2)	U-value
hPD-1.His	1H6	6.44E+05	9.18E-03	1.43E-08	0.05	1
	2E5	5.97E+05	3.66E-03	6.13E-09	0.14	1
	2G4	6.63E+05	4.70E-03	7.09E-09	0.10	1
	2C2	7.33E+05	4.14E-03	5.64E-09	0.03	1
	W3052-16.88.z9-IgG4 (42720)	3.82E+06	1.36E-01	3.56E-08	0.03	5
	WBP305BMK1	4.02E+05	1.35E-03	3.37E-09	0.01	1
	Keytruda	8.79E+05	2.28E-03	2.59E-09	0.07	1

Table 6B

Analyte	Ligand	ka (1/Ms)	kd (1/s)	KD (M)	Chi^2 (RU^2)	U-value
mPD-1.His	1H6	3.20E+05	3.00E-03	9.37E-09	0.06	1
	2E5	3.23E+05	1.29E-03	3.99E-09	0.01	1
	2G4	3.34E+05	1.30E-03	3.89E-09	0.01	1
	2C2	2.21E+05	1.53E-03	6.92E-09	0.19	1
	W3052-16.88.z9-IgG4 (42720)	1.95E+05	8.09E-03	4.16E-08	0.01	1

5. Binding affinity of anti-PD-1 antibodies to cell surface PD-1 molecules tested by flow cytometry (FACS)

[0089] CHO-S cells expressing human PD-1 or 293F cells expressing mouse PD-1 were transferred in to 96-well U-bottom plates (BD) at a density of 1×10^5 cells/well. Testing antibodies were 1:2 serially diluted in wash buffer (1×PBS/1%BSA) and incubated with cells at 4 °C for 1 h. The secondary antibody goat anti-human IgG Fc FITC (3.0 moles FITC per mole IgG, (Jackson Immunoresearch Lab) was added and incubated at 4 °C in the dark for 1 h. The cells were then washed once and resuspended in 1XPBS/1%BSA, and analyzed by flow cytometery (BD). Fluorescence intensity was converted to bound molecules/cell based on the quantitative beads (Quantum™ MESF Kits, Bangs Laboratories, Inc.). KD was calculated using Graphpad PrismS.

[0090] Table.7A-7B show the results of binding affinity of anti-PD-1 antibodies to cell surface human and mouse PD-1 molecules tested by flow cytometry. The results showed that the affinity ability to human PD-1 by FACS assay was from 3.80E-10 to 2.15E-10 mol/L. In addition, the affinity ability to mouse PD-1 was from 5.39E-8 to 1.74E-8 mol/L.

Table 7A

Sample	Best fit-KD (M)
1H6	2.15E-10
2E5	2.30E-10
2G4	3.80E-10

Sample	Best fit-KD (M)
2C2	2.64E-10
W3052-16.88.z9-IgG4 (42720)	4.32E-10
WBP305BMK1	2.62E-10
Keytruda	1.79E-10

Table 7B

Sample	Best fit-KD (M)
1H6	5.39E-08
2E5	2.90E-08
2G4	3.51E-08
2C2	1.74E-08
W3052-16.88.z9-IgG4 (42720)	1.67E-08

6. Epitope binning test

[0091] The binding epitope of anti-PD-1 antibodies was compared with benchmark antibody A and B by FACS. CHO-S cells expressing human PD-1 on the cell surface were incubated with mixture of biotinylated benchmark antibody A or B (1μg/ml) and testing antibodies (serially diluted in wash buffer) at 4 °C for 1 h. The cells were washed and the second antibody Streptavidin-PE were added and incubated for 30 min at 4 °C. The cells were then washed once and resuspended in 1×PBS/1%BSA, and analyzed by flow cytometery (BD).

[0092] Figure 8A-8B showed the results of epitope binning assay suggesting that the anti-PD-1 antibodies are in the same or close epitope bin as benchmark antibodies. Figure.8A showed binning against WBP305BMK1 (US9084776). Figure.8B showed binning against Keytruda (US8168757).

[0093] Furthermore, alanine scanning experiments on hPD-1 were conducted and their effect to antibody binding was evaluated. Alanine residues on hPD-1 were mutated to glycine codons, and all other residues were mutated to alanine codons. For each residue of the hPD-1 extracellular domain (ECD), point amino acid substitutions were made using two sequential PCR steps. A pcDNA3.3-hPD-1_ECD.His plasmid that encodes ECD of human PD-1 and a C-terminal His-tag was used as template, and a set of mutagenic primer was used for first step PCR using the QuikChange lightning multisite-directed mutagenesis kit (Agilent technologies, Palo Alto, CA). Dpn I endonuclease was used to digest the parental template after mutant strand synthesis reaction. In the second-step PCR, linear DNA expression cassette which composed of a CMV promoter, an extracellular domain (ECD) of PD-1, a His-tag and a herpes simplex virus thymidine kinase (TK) polyadenylation was amplified and transiently expressed in HEK293F cells (Life Technologies, Gaithersburg, MD).

[0094] Monoclonal antibodies W3052_r16.88.9 and Keytruda were coated in plates for ELISA binding assay. After interacting with the supernatant that contains quantified PD-1 mutant or human/mouse PD-1_ECD.His protein (Sino Biological, China), HRP conjugated anti-His antibody was added as detection antibody. Absorbance was normalized according to the average of control mutants. After setting an additional cutoff to the binding fold change (<0.55), the final determined epitope residues were identified.

[0095] The binding activities of the antibodies W3052_r16.88.9 and Keytruda to both human and murine PD-1 were conducted (Figure 9). W3052_r16.88.9 was found binding to both hPD-1 and mPD-1 while Keytruda only bound to the human one (Figure 9). This unique functional cross-reactivity of W3052_r16.88.9 can help provide more animal model options in preclinical studies when evaluating the drug safety. To explore the origin of the observed binding behaviors, epitope mapping of both antibodies were conducted.

[0096] Top 30 point-substituted hPD-1 mutants that significantly reduced antibody binding were shown in Table 8. Checking the positions of all these residues on the hPD-1 crystal structures (PDB code 3RRQ and 4ZQK) revealed that some amino acids (e.g. Val144, Leu142, Val110, Met108, Cys123 etc.) were fully buried in the protein, and were unlikely to directly contact any antibodies. The observed binding reductions most probably resulted from the instability or even collapse of hPD-1 structure after alanine substitutions. According to the antigen structure analysis, some of the residues don't involve binding activity, but are expected to respond to the stability of the hPD-1 structure, e.g. V144 and L142. Mutants that affect both antibodies were treated as false hot spots and were removed from the list. After setting an additional cutoff to the binding fold change (<0.55), the final determined epitope residues were listed in Table 9. They are 9 positions to W3052_r16.88.9 and 5 positions to Keytruda.

[0097] Comparing the epitope residues of W3052_r16.88.9 and Keytruda in Table 9 only revealed two overlapped hot spot residues. The rest looked quite diverse, which indicated that two antibodies might have adopted very different mechanisms in terms of hPD-1 binding and hPD-L1 blocking. Reading the residue IDs in Table 9 is not straightforward to interpret the mechanisms. All data in Table 9, as well as the hPD-L1 binding site, were therefore mapped on the crystal structure of hPD-1 to make a better visualization and

comparison. (Figure 10).

Table 8. The effect of PD-1 point mutations on antibody binding

PD-1 #Residue	W3052_r16.88.9		PD-1 #Residue	Keytruda	
	fold change[a]	SD		fold change[a]	SD
V 144	0.09	0.01	P 89	0.18	0.02
L 142	0.21	0.01	D 85	0.38	0.01
K 131	0.27	0.02	V 144	0.4	0.01
P 35	0.31	0	R 94	0.46	0.04
A 129	0.34	0	F 106	0.47	0.05
V 64	0.34	0	K 78	0.48	0
P 83	0.38	0.03	P 83	0.5	0.01
L 128	0.39	0.01	D 92	0.5	0.02
S 137	0.42	0.01	P 39	0.54	0
F 95	0.42	0.01	A 81	0.57	0.01
P 130	0.44	0.01	C 123	0.57	0.01
C 123	0.44	0.01	N 66	0.57	0.03
R 94	0.49	0.04	L 142	0.59	0.01
M 108	0.49	0.02	F 82	0.61	0.03
D 117	0.51	0.01	F 95	0.61	0.04
F 82	0.53	0.02	F 52	0.63	0.01
A 132	0.54	0.02	M 108	0.64	0.06
V 110	0.54	0.02	L 128	0.68	0.01
N 49	0.55	0.01	I 126	0.72	0.01
W 67	0.55	0.01	A 113	0.72	0.01
E 61	0.56	0.04	V 110	0.73	0.04
N 102	0.57	0.04	G 47	0.73	0.01
P 39	0.57	0.01	D 117	0.73	0.07
I 126	0.59	0.04	N 49	0.73	0
A 113	0.6	0.01	S 87	0.74	0.06
F 52	0.61	0.02	L 42	0.76	0.01
H 155	0.62	0.04	N 102	0.76	0.01
R 86	0.64	0.08	W 67	0.81	0.01
A 149	0.64	0	P 101	0.81	0.04
G 47	0.64	0.03	A 80	0.82	0.01

[a] Fold change in binding is relative to the binding of several silent alanine substitutions.

Table 9. Identification of potential epitopes

PD-1 to r16.88.9	residue location	PD-1 to Keytruda		residue location	
P 35	A	K 78		C'	
V 64	C	P 83		C'	
F 82	C'	D 85		C"	
P 83	C'	P 89		C"	
L 128	FG	D 92		C"D	
A 129	FG				
P 130	FG				
K 131	FG				
A 132	FG				

Cutoff: fold change<0.55
* The C" strand observed on mPD-1 does not exist on hPD-1 structure. This β-sheet is replaced by a structreless loop on hPD-1. We still use C" to label this region, just for the purpose of easier comparison to mPD-1.

[0098] Two investigated antibodies W3052_r16.88.9 and Keytruda, although both are functional in binding hPD-1 and blocking hPD-L1,

have obviously different epitopes (Figure 10B, 10C). The epitope of Keytruda were mainly contributed by the residues on the C'D loop (corresponding to the C" strand on mPD-1), which didn't intersect the PD-L1 binding site at all. This suggested the hPD-L1 blocking function of Keytruda relied more on its steric hindrance effects provided by the size of the antibody. In contrast, the epitope mapping results show that the epitope of antibody W3052_r16.88.9 was composed of hot spots distributed across multiple locations, and have direct overlap with the hPD-L1 binding site (Figure 10A, 10B). W3052_r16.88.9 blocked hPD-L1 by means of competing with hPD-L1 in reacting to their common binding site. What's more, W3052_r16.88.9 had no interactions with the flexible C'D loop (or the corresponding C" strand on mPD-1), where human and murine PD-1 show big structural deviations (Figure 11). Its binding site is mostly located on the FG loop (Lin et al. (2008) PNAS 105: 3011-3016). That explains why W3052_r16.88.9 can bind to both PD-1 species while Keytruda only binds to the human one (Figure 9). Because of this unique functional cross-reactivity, the preclinical safety evaluations of W3052_r16.88.9 could be conducted in mouse model, which will greatly simplify and accelerate the development. Overall, antibody W3052_r16.88.9 is expected to be more functional and developable than Keytruda.

7. In vitro function of anti-PD-1 antibodies tested by cell-based assays

7.1 Mixed Lymphocyte Reaction (MLR) was used to test the effects of anti-PD-1 antibodies on T lymphocytes function

[0099] Human DCs, $CD4^+$ T, $CD8^+$ T and total T cells isolation: Human PBMCs were freshly isolated from healthy donors using Ficoll-Paque PLUS (GE) gradient centrifugation. Monocytes were isolated using Human Monocyte Enrichment Kit (StemCell) according to the manufacturer's instructions. Cells were cultured in medium containing rhGM-CSF and rhIL-4 for 5 to 7 days to generate dendritic cells. 18 to 24 hours before MLR, 1 μg/mL LPS was added to the culture to induce the maturation of the DCs. Human $CD4^+$ T cells were isolated using Human $CD4^+$ T Cell Enrichment Kit (StemCell) according to the manufacturer's protocol. Mouse $CD4^+$ T cells were obtained from the spleen of *Balb/c* mouse using Mouse $CD4^+$ T Cell Isolation Kit (StemCell) according to the manufacturer's protocol. Mouse DCs were induced from bone marrow cells of C57BL/6 mouse in medium containing rmGM-CSF and rmIL-4 for 5 to 7 days. 18 to 24 hours before MLR, 1 μg/mL LPS was added to the culture to induce the maturation of the DCs.

[0100] Briefly, primary dendritic cell (DC)-stimulated MLR was conducted in 96-well, U-bottom tissue culture plates in 200 μL of RPMI 1640 containing 10% FCS and 1% antibiotics. DCs were mixed with 1×10^5 $CD4^+$ T cells at a ratio between 1:10 and 1:200 DC: T cells in the presence or absence of testing antibodies or benchmark antibodies (form 166.75 nM down to 0.00667 nM, generally total six concentrations). To determine the effect of anti-PD-1 antibodies on T cell function, the cytokine production and T cell proliferation were determined. Results shown are representative of a minimum of five experiments performed.

[0101] Cytokine detection: Human IFN-γ and IL-2 were measured by enzyme-linked immunosorbent assay (ELISA) using matched antibody pairs. The plates were pre-coated with capture antibody specific for human IFN-γ (cat# Pierce-M700A) or IL-2 (cat# R&D-MAB602), respectively. The biotin-conjugated anti-IFN-γ antibody (cat# Pierce-M701B) or anti-IL-2 antibody (cat# R&D-BAF202) was used as detecting antibody.

[0102] Figure 12A showed anti-PD-1 antibodies increased IL-2 secretion in a dose-dependent manner. Figure 12B shows anti-PD-1 antibodies increase IFN-γ secretion in a dose-dependent manner.

[0103] Proliferation assay: 3H-thymidine (cat# PerkinElmer- NET027001MC) was diluted 1:20 in 0.9% NaCl solution, and added to the cell culture plates at 0.5 uCi/well. The plates were cultured in 5% CO_2 at 37°C for 16 to 18 hours, before the incorporation of 3H-thymidine into the proliferating cells was determined. Figure 12C shows anti-PD-1 antibodies increase $CD4^+$ T cells proliferation in a dose-dependent manner.

[0104] To determine the effect of anti-PD-1 antibodies on mouse T cell function, the cytokine production and mouse T cell proliferation were determined similarly. Figure 13A-13C showed the results of mouse allo-MLR demonstrating that the anti-PD-1 antibodies can enhance the function of mouse $CD4^+$ T cell. Figure 13A showed anti-PD-1 antibodies increased IL-2 secretion in a dose-dependent manner. Figure. 13B showed anti-PD-1 antibodies increased IFN-γ secretion in a dose-dependent manner. Figure 13C showed anti-PD-1 antibodies increased $CD4^+$ T cells proliferation in a dose-dependent manner.

7.2 Effect of human anti-PD-1 antibodies on cell proliferation and cytokine production by autologous antigen specific immune response

[0105] In this assay, the $CD4^+$ T cells and DCs were from a same donor. Briefly, $CD4^+$ T cells were purified from PBMC and cultured in the presence of CMV pp65 peptide and low dose of IL-2 (20 U/mL), at the meanwhile, DCs were generated by culturing monocytes from the same donor's PBMC in GM-CSF and IL-4. After 5 days, the CMV pp65 peptide treated $CD4^+$ T cells were co-cultured with DCs

pulsed with CMV pp65 peptide in the absence or presence of human anti-PD-1 antibodies or benchmark antibodies (as control). On day 5, 100 µL of supernatants were taken from each of cultures for IFN-γ measurement by ELISA as described above. The proliferation of CMV pp65-specific T cells was assessed by 3H-thymidine incorporation as described above.

[0106] Figure 14A-14B showed the results of human auto-MLR demonstrating the anti-PD-1 antibodies can enhance the function of human $CD4^+$ T cell. Figure 14A showed anti-PD-1 antibodies increase IFN-γ secretion in a dose-dependent manner. Figure 14B showed anti-PD-1 antibodies increase $CD4^+$ T cells proliferation in a dose-dependent manner.

7.3 Effect of human anti-PD-1 antibodies on regulatory T cell (Tregs) suppressive function

[0107] Tregs, a subpopulation of T cells, are a key immune modulator and play critical roles in maintaining self-tolerance. Increased numbers of $CD4^+CD25^+$ Tregs were found in patients with multiple cancers and associated with a poorer prognosis. To determine whether the anti-PD-1 antibodies affect the immune suppressive role of Tregs, we compared the T cell function in the presence of Tregs with or without anti-PD-1 antibody treatment. $CD4^+CD25^+$ and $CD4^+CD25^-$T cells were separated using specific anti-CD25 microbeads (StemCell) per manufacture's instruction. Two thousand mature DCs, 1×10^5 $CD4^+CD25^-$ T cells, 1×10^5 Treg cells and PD-1 antibodies were incubated in 96-well plates. The plates were kept at 37°C in a 5% CO_2 incubator for 5 days. IFN-γ production and $CD4^+CD25^-$ cells proliferation were tested as described above.

[0108] Figure 15 demonstrates that the anti-PD-1 antibodies can reverse the suppressive function of Tregs. Figure 15A showed anti-PD-1 antibodies can restore the IFN-γ secretion. Figure 15B showed anti-PD-1 antibodies can restore the T-cell proliferation.

8. ADCC and CDC test

[0109] PD-1 is expressed on variety of cell types. In order to minimize potential toxicity to healthy PD-1 positive cells, the anti-PD-1 antibodies were evaluated for their ability to mediate antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC).

8.1 ADCC test

[0110] Human activated $CD4^+$ T cells and various concentrations of PD-1 antibodies were pre-incubated in 96-well plate for 30 minutes, and then PBMCs were added at the effector/target ratio of 50:1. The plate was kept at 37 °C in a 5% CO_2 incubator for 6 hours. Target cell lysis was determined by LDH-based cytotoxicity detection kit (cat# Roche-11644793001). The absorbance at 492 nm was read using a microplate reader (Molecular Device). Herceptin-induced SK-Br-3 cell lysis was used as positive control.

[0111] Figure 16 showed the result of ADCC test demonstrating the anti-PD-1 antibodies did not mediate ADCC activity on activated $CD4^+$ T cells.

8.2 CDC test

[0112] Human activated $CD4^+$ T cells and various concentrations of PD-1 antibodies were mixed in 96-well plate. Human complement (Quidel-A112) was added at the dilution ratio of 1:50. The plate was kept at 37 °C in a 5% CO_2 incubator for 2 hours. Target cell lysis was determined by CellTiter-Glo. Rituxan®-induced Raji cell lysis was used as positive control. The luminescence was read using a microplate reader (Molecular Device).

[0113] Figure 17 showed the result of CDC test demonstrating the anti-PD-1 antibodies did not mediate CDC activity on activated $CD4^+$ T cells.

Example 5: Treatment of *in vivo* tumor model using human monoclonal antibodies against PD-1

1. Experimental Design

[0114]

Table 10 Grouping and dosing regimen of the *in vivo* animal efficacy experiments of antibody 2E5

group	N[1]	Treatment	Dose (mg/kg)	Dose-Volume Parameters (μl/g)[2]	Route of administration	Frequency of administration
1	6	Vehicle	--	10	IP	Q3D×5
3	6	2E5	1 mg/kg	10	IP	Q3D×5
4	6	2E5	3 mg/kg	10	IP	Q3D×5
5	6	2E5	10 mg/kg	10	IP	Q3D×5

Annotations:

[0115]

[1]N: mice number in each group

[2]Dose-Volume: 10 μL/g according to the weight of mouse. If the weight loss exceeds 15%, the dosing regimen should be adjusted accordingly.

2. Methods

2.1 Cell Culture

[0116] Murine melanoma cell CloudmanS91 cell (ATCC-CCL-53.1) was cultured in vitro as monolayer, and the culture condition was F-12K medium plus 2.5% FBS and 15% horse serum, 100 U/mL penicillin, and 100 μg/mL streptomycin, incubate at 37 °C and 5% CO_2. The cells were digested using trypsin-EDTA and passaged twice a week routinely. Cells were harvested, counted, and then inoculated when approximately 80%-90% confluent and the number is as required.

2.2 Injection of tumor cells

[0117] 0.1 mL (5×10^5 cells) CloudmanS91 cells were inoculated subcutaneously in the right backside of each animal. When the mean of tumor volume had reached approximately 64 mm^3, the administration started in groups. Grouping and dosing regimens were shown in Table 10.

2.3 Tumor testing and Index

[0118] Experimental index is to investigate whether the tumor growth was inhibited, delayed or cured. Tumor diameters were measured with a caliper three times a week. Tumor volume is calculated using $V=0.5a\times b^2$, wherein *a* and *b* represents long and short diameters of the tumor, respectively.

[0119] Antitumor efficacy of the antibody was assessed by tumor growth inhibition TGI (%) or relative tumor proliferation rate T/C (%). TGI (%) reflected the rate of tumor growth inhibition. TGI (%) was calculated as follows: TGI (%) = [(1-(average tumor volume at the end of administration in the treatment group - average tumor volume at the start of administration in the treatment group)) / (average tumor volume at the end of treatment in the solvent control group - average tumor volume at the start of treatment in the solvent control group)] × 100%.

[0120] Relative tumor proliferation rate T/C (%) was calculated as follows: $T/C\% = T_{RTV} / C_{RTV} \times 100\%$ (T_{RTV}: treatment group RTV; C_{RTV}: negative control group RTV). The relative tumor volume (RTV) was calculated according to the results of tumor measurements using $RTV=V_t/V_0$, wherein V_0 was average tumor volume at the time of grouping (i.e., d_0), V_t was average tumor volume of a certain measurement; the data of T_{RTV} and C_{RTV} were taken on the same day.

[0121] T-C (days) reflected tumor growth delay index, T represented average days passed when the tumor had reached a predetermined volume in the treatment group (eg. 300 mm^3), C represented the average days when tumors in the control group had

reached the same volume.

[0122] Survival curves were plotted; animal survival time was defined as the time from the administration to animal deaths or the time when tumor volume had reached 2000 mm^3. The median survival time (days) was calculated in each group. Increased life span (ILS) was calculated by comparison of the median survival times between the treated group and model control group and represented as a percentage over the lifetime of the model control group.

2.4 Statistical Analysis

[0123] The data including the average tumor volume at each time point in each group and standard error (SEM) were analyzed statistically (refer to Table 11 for specific data). The experiment was completed on day 37 after the administration; on day 13 after the administration, start sacrificing animals successively; and therefore the statistical analysis and evaluation for inter-group differences were based on the tumor volume on day 13 after initiation of administration. For comparisons between the two groups, data were analyzed using T-test; for comparisons among three or more groups, data were analyzed using one-way ANOVA. If statistically significant difference was found for F value, data were analyzed using Games-Howell test. If no statistically significant difference was found for F value, Dunnet (2-sided) test was then used for analysis. SPSS 17.0 was used for all data analysis. $p<0.05$ was considered as significant difference. Survival time was analyzed using Kaplan-Meier method with the Log-rank test.

3. Results

3.1 Mortality, morbidity and body weight changes

[0124] Animal's weight is as an indirect reference for measurement of drug toxicity. The impact of 2E5 on the weight of CloudmanS91 subcutaneous syngeneic xenograft female DBA/2 mice model was as shown in Figure 18 and Figure 19. In this model, all administration groups showed no significant weight loss (Figure 18). Thus, 2E5 had no obvious toxicity in a mouse model of melanoma CloudmanS91.

3.2 Tumor volume

[0125] Tumor volume in CloudmanS91 subcutaneously syngeneic xenograft female DBA/2 mouse model after 2E5 treatment was as shown in Table 11.

Table 11 Tumor volume at different times in each group

Days	Tumor volume (mm^3)[a]			
	Vehicle	2E5 1 mg/kg	2E5 3 mg/kg	2E5 10 mg/kg
0[b]	66 ± 9	65 ± 8	64 ± 8	63 ± 8
2	142 ± 23	129 ± 10	110 ± 10	94 ± 8
4	251 ± 39	231 ± 34	162 ± 9	143 ± 17
6	345 ± 65	339 ± 61	200 ± 13	197 ± 38
9	599 ± 66	597 ± 100	281 ± 38	291 ± 83
11	1,026 ± 173	943 ± 307	335 ± 66	475 ± 190
13	1,626 ± 262	1,089 ± 365	361 ± 81	614 ± 273

Annotations:
a. average ± SEM;
b. Days after administration.

3.3 Tumor growth curve

[0126] Tumor growth curve was shown in Figure 20.

3.4 Antitumor efficacy evaluation

[0127]

Table 12 Antitumor efficacy evaluation of 2E5 in CloudmanS91 Syngeneic tumor model (based on the tumor volume on day 13 after initiation of administration)

group	Tumor volume (mm^3)[a] (Day 13)	T/C [b] (%)	TGI [b] (%)	T-C (Days) (300 mm^3)	*p* value [c]
Vehicle	1,626 ± 262	--	--	--	--
2E5 (1 mg/kg)	1,089 ± 365	68.1	34.4	0	0.367
2E5 (3 mg/kg)	361 ± 81	22.9	81.0	5	0.008
2E5 (10 mg/kg)	614 ± 273	39.4	64.7	5	0.036
Annotations: a . average ± SEM; b. Tumor growth inhibition was calculated by T/C and TGI (TGI (%) = $[1-(T_{13}-T_0)/(V_{13}-V_0)] \times 100$); c. p value was calculated by tumor volume.					

3.5 Survival curves

[0128] Survival curves in each group were shown in Figure 21.

3.6 Survival time

[0129]

Table 13 Effect of 2E5 on survival of CloudmanS91 Syngeneic tumor model

group	Median survival time (Days)	Prolonged survival (%)	Log Rank P value[a]
Vehicle	16	-	-
2E5 1 mg/kg	20	25	0.077
2E5 3 mg/kg	N/A[b]	N/A	0.001
2E5 10 mg/kg	32	100	0.022
Annotations: a. p-value represented the comparison between the treatment group and the vehicle control group; b. At the end of the experiment, in 3mg/kg group (2E5), the survival rate was 66.7%.			

4. Discussion

[0130] In this study, we have evaluated the in vivo efficacy of 2E5 in CloudmanS91 syngeneic tumor model. Tumor volume in each group at different time points were shown in Table 11, Table 12 and Figure 20, survival time were shown in Figure 21 and Table 13. On day 13 after administration, tumor volume of tumor-bearing mice in the solvent control group reached 1,626 mm^3. A weak inhibitory effect was observed in 1 mg/kg 2E5 group compared with the control group, and the tumor volume was 1,089 mm^3 (T/C=68.1%, TGI=34.4%, p=0.367), tumor growth delay was 0 days. A significant anti-tumor effect was observed in 3 mg/kg 2E5 group compared with the solvent control group, and the tumor volume was 361 mm^3 (T/C=22.9%, TGI=81.0%, p=0.008), tumor growth delay was 5 days. A significant anti-tumor effect was also observed in 10 mg/kg 2E5 group compared with the solvent control group, the tumor volume was 614 mm^3 (T/C=39.4%, TGI=64.7%, p=0.036), tumor growth delay was 5 days.

[0131] In the experiment, the median survival time of tumor-bearing mice in solvent control group was 16 days. Compared with the vehicle control group, the median survival time of tumor-bearing mice in 1 mg/kg 2E5 group was 20 days, survival was prolonged 25% (p=0.077); survival rate of tumor-bearing mice in 3 mg/kg 2E5 group was 66.7% (p=0.001). The median survival time of tumor-bearing mice in 10 mg/kg 2E5 group was 32 days, survival was prolonged 100% (p=0.022).

[0132] The changes in body weight of nude mice were shown in Figure 19. Good tolerability of drug 2E5 has been found in all tumor-bearing mice, and no significant weight loss was observed in all treatment groups. In summary, in this experiment, significant anti-tumor effects were shown in both 3 mg/kg group and 10 mg/kg group for CloudmanS91 subcutaneous synergistic tumor model, which is not dose-dependent. Anti-tumor effect in 3 mg/kg group is better than that in 10 mg/kg group.

[0133] The description of the present invention has been made above by the examples. However, it is understood by the skilled in the art that the present invention is not limited to the examples. Scope of the invention is thus indicated by the appended claims rather than

by the foregoing description.

REFERENCES CITED IN THE DESCRIPTION

Cited references

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO2016077397A [0007]
- US9084776B [0040] [0092]
- US8168757B [0040] [0092]
- US9084776B2 [0088]

Non-patent literature cited in the description

- **OKAZAKI et al.**Curr. Opin. Immunol., 2002, vol. 14, 391779-82 [0002]
- **BENNETT et al.**J Immunol, 2003, vol. 170, 711-8 [0002] [0004]
- **OKAZAKI, TAKU et al.**International Immunology, 2007, vol. 19, 813-824 [0002]
- **ISHIDA et al.**EMBO J, 1992, vol. 11, 3887-95 [0002]
- **AGATA et al.**bit Immunol, 1996, vol. 8, 765-72 [0003]
- **FREEMAN et al.**J Exp Med, 2000, vol. 192, 1027-34 [0003]
- **LATCHMAN et al.**Nat Immunol, 2001, vol. 2, 261-8 [0003]
- **CARTER et al.**Eur J Immunol, 2002, vol. 32, 634-43 [0003]
- **OKAZAKI et al.**Curr Opin Immunol, 2002, vol. 14, 391779-82 [0004]
- **NISHIMURA et al.**Immunity, 1999, vol. 11, 141-51 [0004]
- **NISHIMURA et al.**Science, 2001, vol. 291, 319-22 [0004]
- **SALAMA et al.**J Exp Med, 2003, vol. 198, 71-78 [0004]
- **PROKUNINAALARCON-RIQUELME**Hum Mol Genet, 2004, vol. 13, R143- [0004]
- **NIELSEN et al.**Lupus, 2004, vol. 11, 510- [0004]
- **OKAZAKI et al.**PNAS, 2001, vol. 98, 13866-71 [0004]
- **DONG et al.**Nat. Med, 2002, vol. 8, 787-9 [0005]
- **DONG et al.**J. Mol. Med., 2003, vol. 81, 281-7 [0005]
- **BLANK et al.**Cancer Immunol. Immunother., 2005, vol. 54, 307-314 [0005]
- **KONISHI et al.**Clin. Cancer Res., 2004, vol. 10, 5094-100 [0005]
- **C. WANG et al.**Cancer Immunology Research, 2014, vol. 2, 9846-856 [0006]
- **LIN et al.**PNAS, 2008, vol. 105, 3011-3016 [0098]

PATENTKRAV

1. Et antistof eller et antigenbindende fragment deraf, der specifikt binder til PD-1, hvor antistoffet eller det antigenbindende fragment deraf omfatter:

a) en variabel region af en tung kæde med aminosyresekvensen i SEQ ID NO: 1 og en variabel region af en let kæde med aminosyresekvensen i SEQ ID NO: 3;

b) en variabel region af en tung kæde med aminosyresekvensen SEQ ID NO: 2 og et variabel område af en let kæde med aminosyresekvensen SEQ ID NO: 3;

c) en variabel region af en tung kæde med aminosyresekvensen i SEQ ID NO: 2 og en variabel region af en let kæde med aminosyresekvensen i SEQ ID NO: 4; eller

d) en variabel region af en tung kæde med aminosyresekvensen i SEQ ID NO: 2 og en variabel region af en let kæde med aminosyresekvensen i SEQ ID NO: 5.

2. Antistoffet eller antigenbindende fragment deraf ifølge krav 1, hvor PD-1 er murin PD-1, som er mus eller rotte PD-1.

3. Antistoffet eller antigenbindende fragment deraf ifølge krav 1 eller 2, hvor antistoffet eller det antigenbindende fragment deraf

a) binder til humant PD-1 med en KD på 2,15E-10 M eller mindre ved FACS-analyse; og

b) binder til mus-PD-1 med en KD på 1,67E-08 M eller mindre ved FACS-analyse; og hvor antistoffet udviser mindst en af følgende egenskaber:

c) binder til humant PD-1 med en KD på mellem 4,32E-10 M og 2,15E-10 M og til muse-PD-1 med en KD på mellem 5,39E-08 M og 1,67E-08 M ved FACS-analyse;

d) binder ikke specifikt til human CD28, CTLA-4;

e) øger T-celleproliferation;

f) øger produktionen af interferon-gamma i T-celler; eller

g) øger interleukin-2-sekretionen i T-celler.

4. Antistoffet eller antigenbindende fragment deraf ifølge et hvilket som helst af kravene 1 til 3, hvor antistoffet er et humaniseret antistof.

5. Antistoffet eller antigenbindende fragment deraf ifølge et hvilket som helst af kravene 1-4, hvor antistoffet udviser mindst en af følgende egenskaber:

a) binder til humant PD-1 med en KD på 2,15E-10 M eller mindre og til mus-PD-1 med en KD på 1,67E-08 M eller mindre ved FACS-analyse;

b) binder ikke specifikt til humant CD28, CTLA-4;

c) øger T-celleproliferation;

d) øger produktionen af interferon-gamma i T-celler; eller

e) øger interleukin-2-sekretionen i T-celler.

6. Et nukleinsyremolekyle, der koder for antistoffet eller det antigenbindende fragment ifølge et hvilket som helst af kravene 1 til 5.

7. En klonings- eller ekspressionsvektor, der omfatter nukleinsyremolekylet ifølge krav 6.

8. En værtscelle, der omfatter en eller flere klonings- eller ekspressionsvektorer ifølge krav 7.

9. Fremgangsmåde til fremstilling af antistoffet ifølge et hvilket som helst af kravene 1 til 5, omfattende dyrkning af værtscellen i krav 8 og isolering af antistoffet; hvor antistoffet fortrinsvis fremstilles gennem immunisering i SD-rotte med humant PD-1 ekstracellulært domæne og mus PD-1 ekstracellulært domæne.

10. En transgen mus, der omfatter humane immunoglobulintunge og lette kædetransgener, hvor musen udtrykker antistoffet ifølge et hvilket som helst af kravene 1 til 5.

11. Et hybridom fremstillet af musen ifølge krav 10, hvor hybridomet producerer det nævnte antistof.

12. En farmaceutisk sammensætning, der omfatter antistoffet eller det antigenbindende fragment ifølge et hvilket som helst af kravene 1 til 5 og et eller flere af et farmaceutisk acceptabelt hjælpestof, fortyndingsmiddel eller bærestof.

13. Immunokonjugat, der omfatter antistoffet eller det antigenbindende fragment heraf ifølge et hvilket som helst af kravene 1 til 5, bundet til et terapeutisk middel.

14. En farmaceutisk sammensætning, der omfatter immunokonjugatet ifølge krav 13 og et farmaceutisk acceptabelt hjælpestof, fortyndingsmiddel eller bærestof.

15. Et antistof ifølge et hvilket som helst af kravene 1 til 5 til brug i en metode til behandling eller profylakse af en immunforstyrrelse eller kræft.

16. Antistoffet til brug ifølge til krav 15 i en metode til behandling eller forebyggelse af kræft ved at hæmme væksten af tumorceller i et individ, omfattende indgivelse til individet af en terapeutisk effektiv mængde af antistoffet for at hæmme væksten af tumorcellerne i en kræftsygdom.

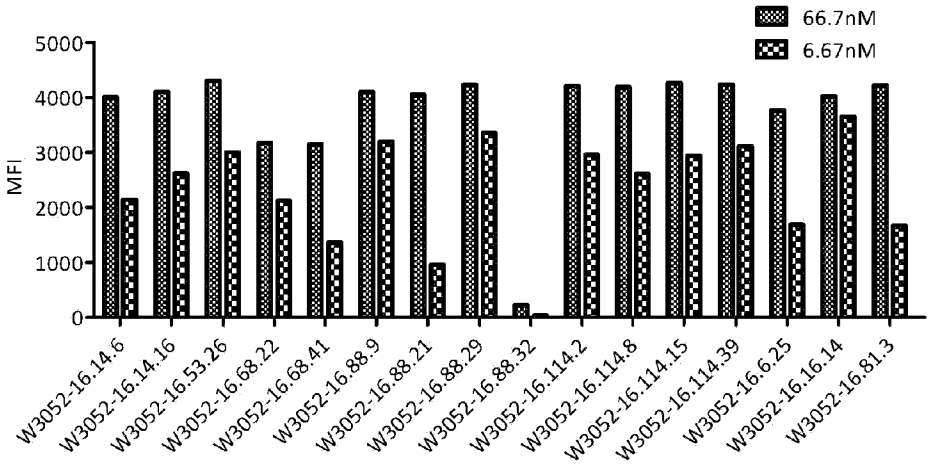
17. Antistoffet til brug ifølge krav 16, hvor kræften er valgt fra en gruppe bestående af melanom, nyrekræft, prostatakræft, brystkræft, tyktarmskræft, lungekræft, knoglekræft, bugspytkirtelkræft, hudkræft, kræft i hoved eller hals, kutan eller intraokulær malignt melanom, livmoderkræft, kræft i æggestokkene og endetarmskræft.

18. Antistof til brug ifølge krav 16 eller 17, hvor antistoffet er et humaniseret antistof.

DRAWINGS

Drawing

A



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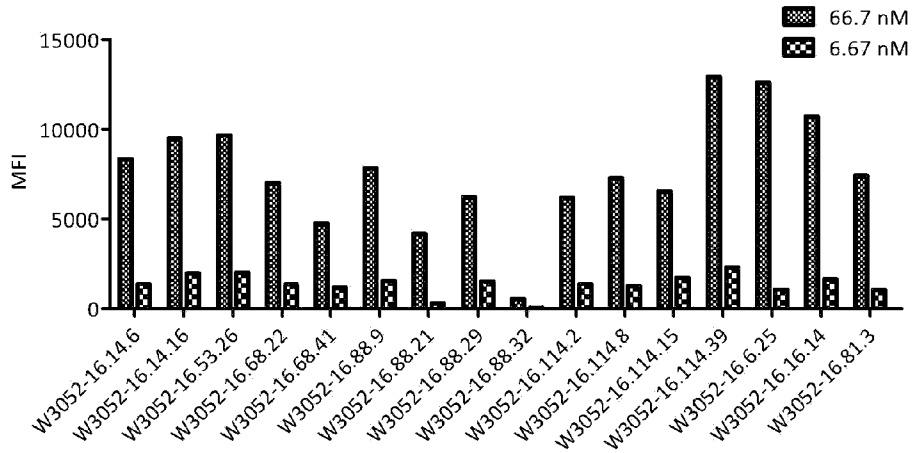
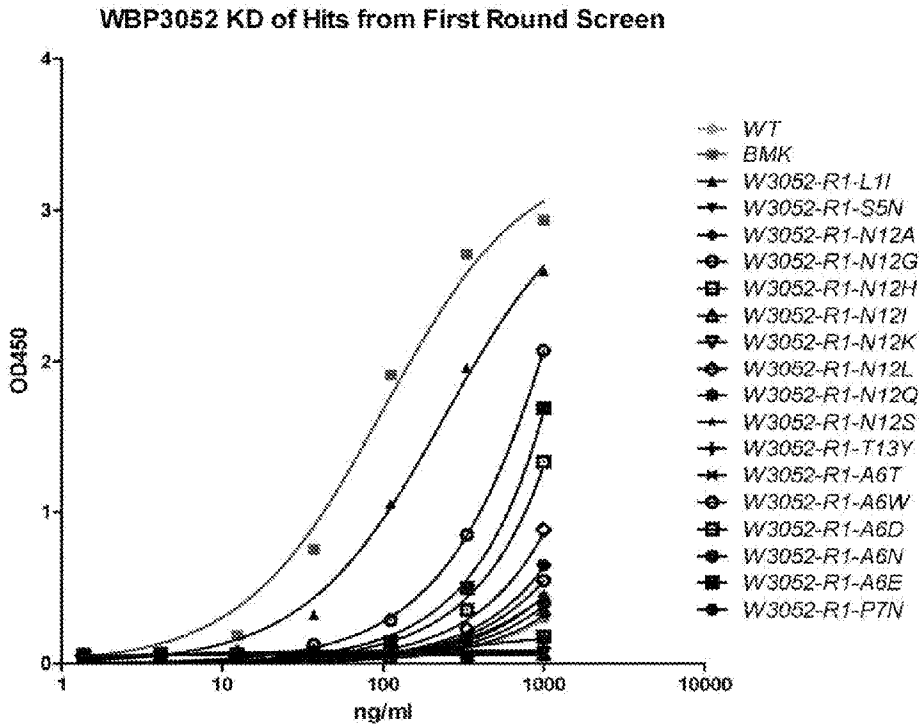


Figure 1


WBP3052 KD of Hits from First Round Screen
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ng/ml
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1
2
3
4
1
10
100
1000
10000
WT
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W3052-R1-S5N
W3052-R1-N12A
W3052-R1-N12G
W3052-R1-N12H
W3052-R1-N12I
W3052-R1-N12K
W3052-R1-N12L
W3052-R1-N12Q
W3052-R1-N12S
W3052-R1-T13Y
W3052-R1-A6T
W3052-R1-A6W
W3052-R1-A6D
W3052-R1-A6N
W3052-R1-A6E
W3052-R1-P7N

Figure 2

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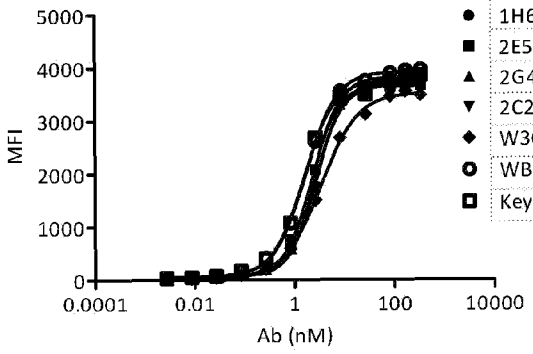
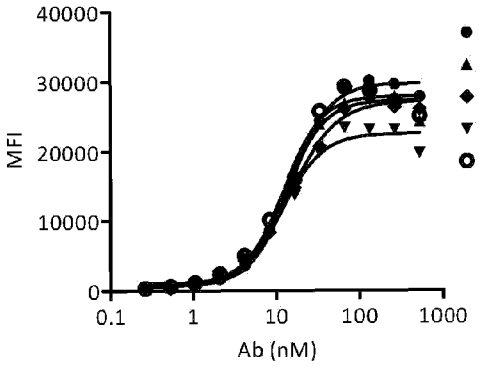


Abs	EC50 (nM)
1H6	2.64
2E5	2.20
2G4	2.78
2C2	2.36
W3052-16.88-z9-IgG4 (42720)	3.44
WBP305BMK1	1.69
Keytruda	1.54

B



Abs	EC50 (nM)
1H6	15.1
2E5	12.9
2G4	14.8
2C2	11.8
W3052-16.88-z9-IgG4 (42720)	12.5

C

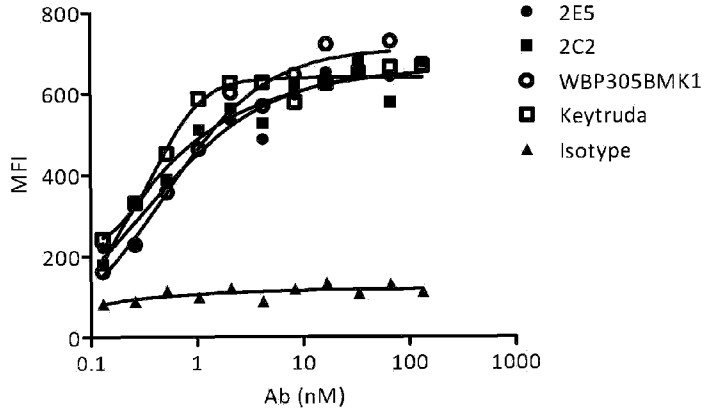
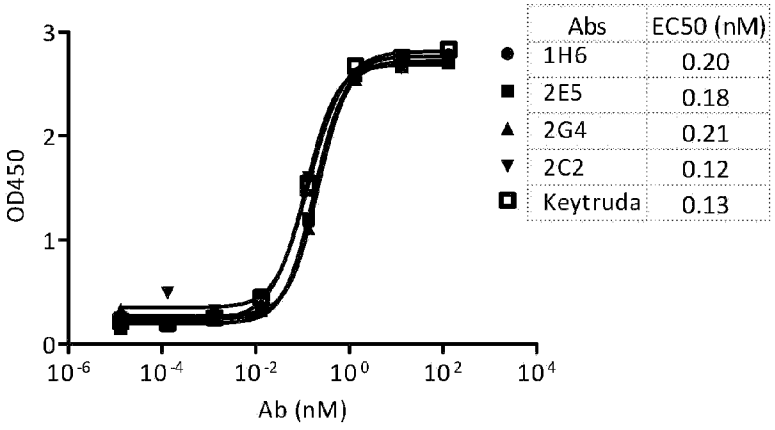


Figure 3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Ab (nM)
Abs
EC50 (nM)
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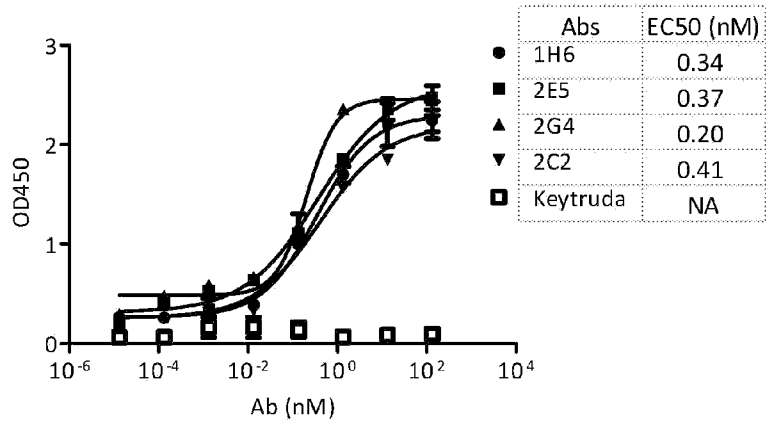
2G4
0.21
2C2
0.12
Keytruda
0.13

B


OD450
Ab (nM)
Abs
EC50 (nM)
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2G4
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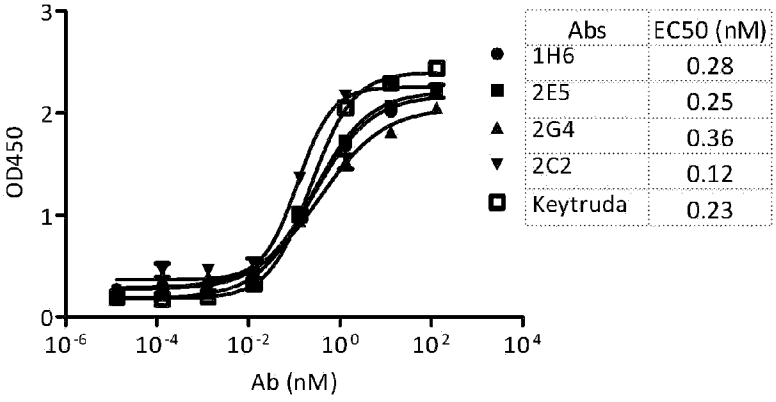
2C2
0.41
Keytruda
NA

C


OD450
Ab (nM)
Abs
EC50 (nM)
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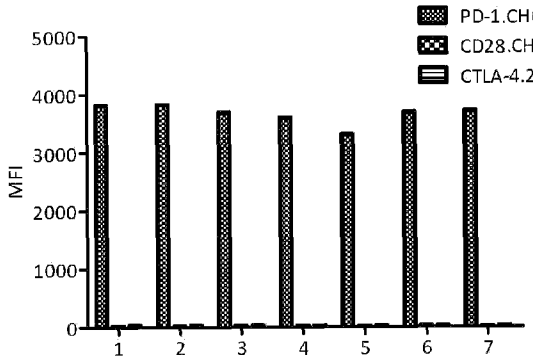
2E5
0.25
2G4
0.36
2C2
0.12
Keytruda
0.23

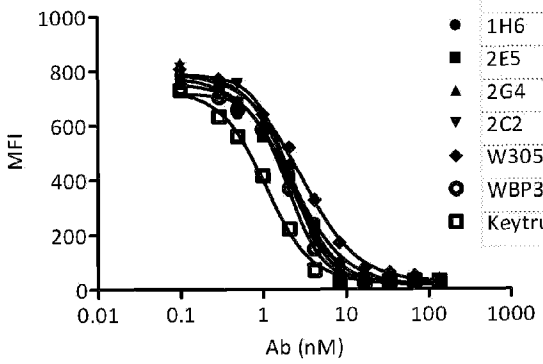
Figure 4



NO.	Antibody
1	1H6
2	2E5
3	2G4
4	2C2
5	W3052-16.88-z9-IgG4 (42720)
6	WBP305BMK1
7	Keytruda

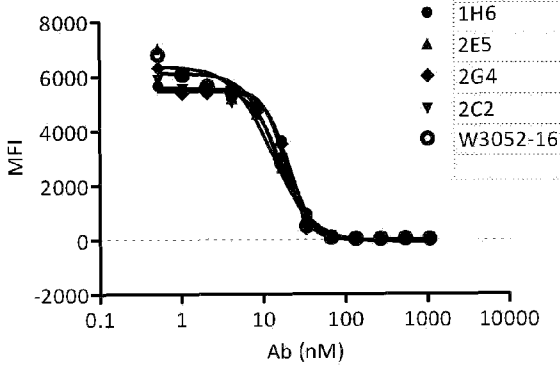
Figure 5

A



Abs	IC50 (nM)
1H6	2.22
2E5	2.14
2G4	2.23
2C2	2.20
W3052-16.88-z9-IgG4 (42720)	2.95
WBP305BMK1	2.03
Keytruda	1.09

B



Abs	IC50 (nM)
1H6	20.4
2E5	13
2G4	18.7
2C2	16.7
W3052-16.88-z9-IgG4 (42720)	14.8

Figure 6

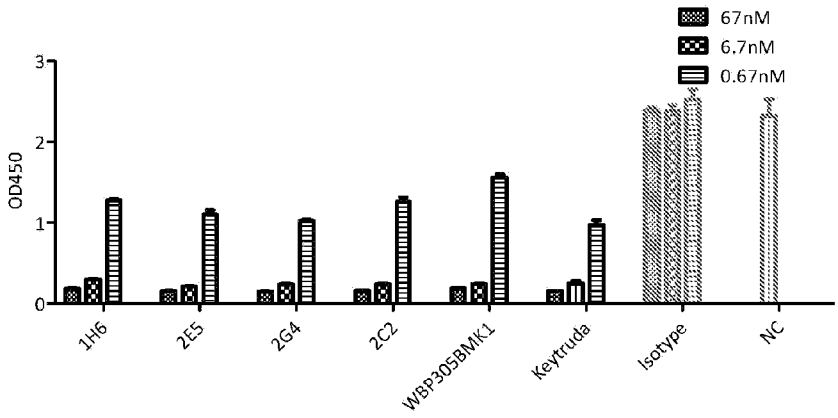


Figure 7

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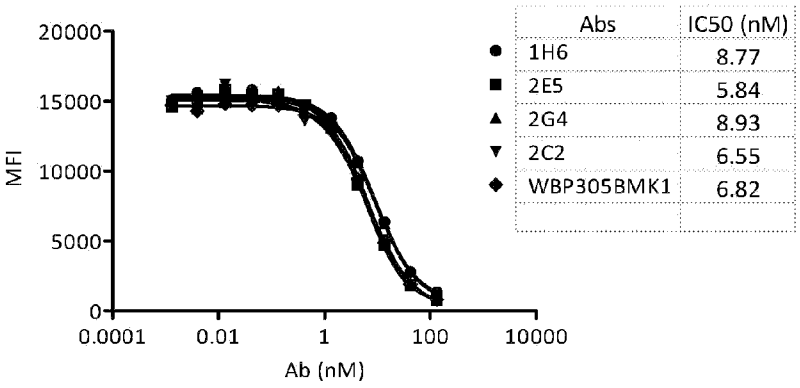

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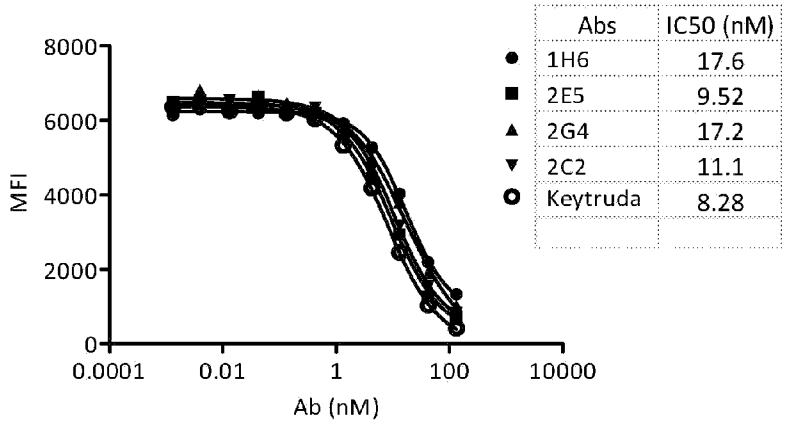


Figure 8B

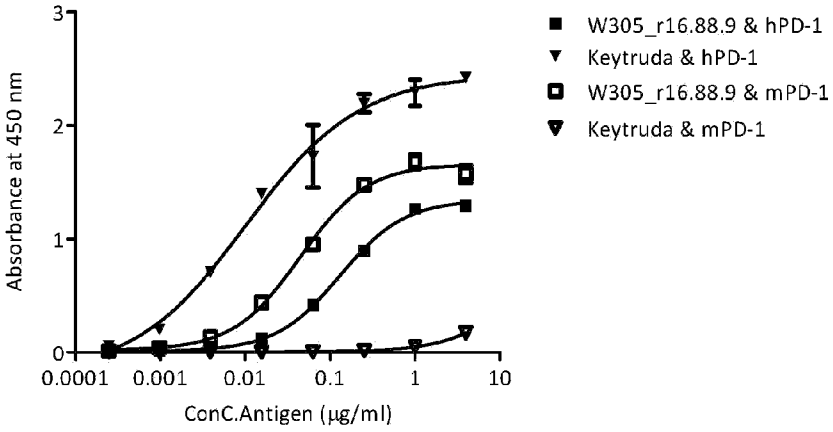

Absorbance at 450 nm
3
2
1
0
0.0001
0.001
0.01
0.1
1
10
ConC.Antigen (μg/ml)
W305_r16.88.9 & hPD-1
Keytruda & hPD-1
W305_r16.88.9 & mPD-1
Keytruda & mPD-1

Figure 9

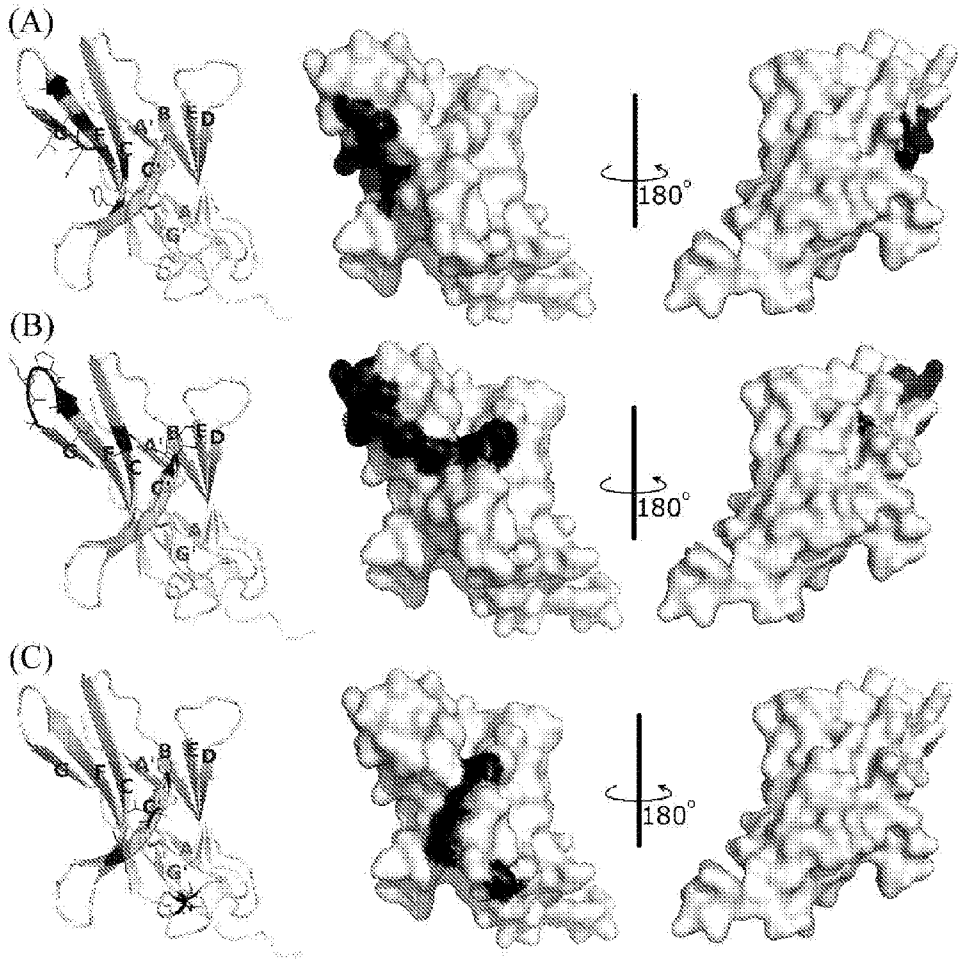


Figure 10

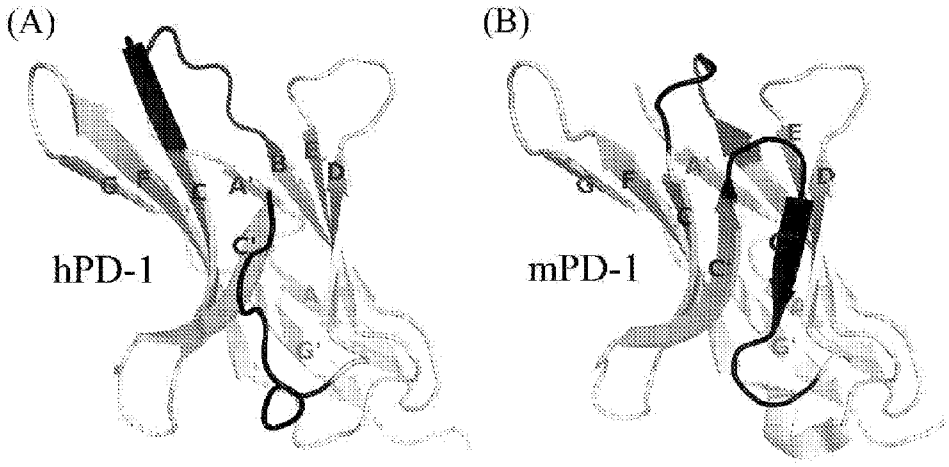
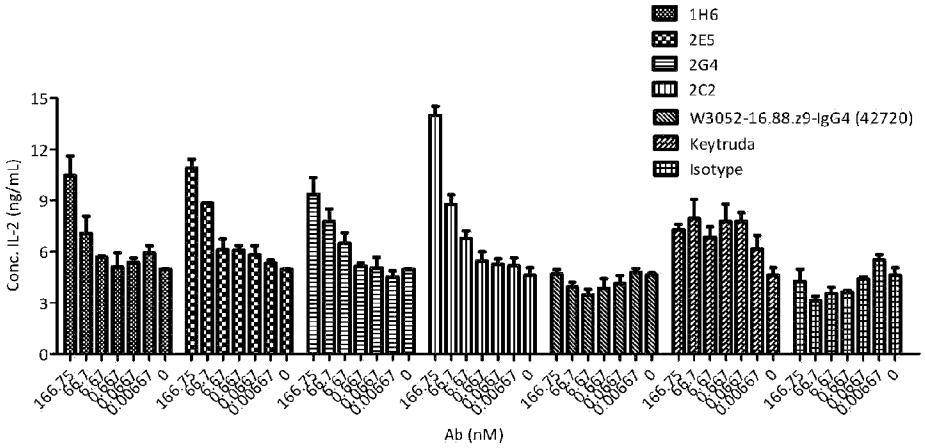
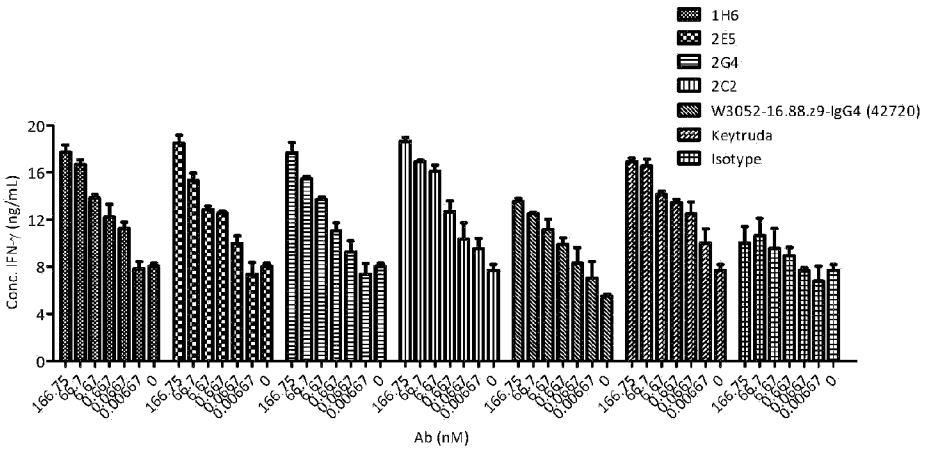


Figure 11

A



B



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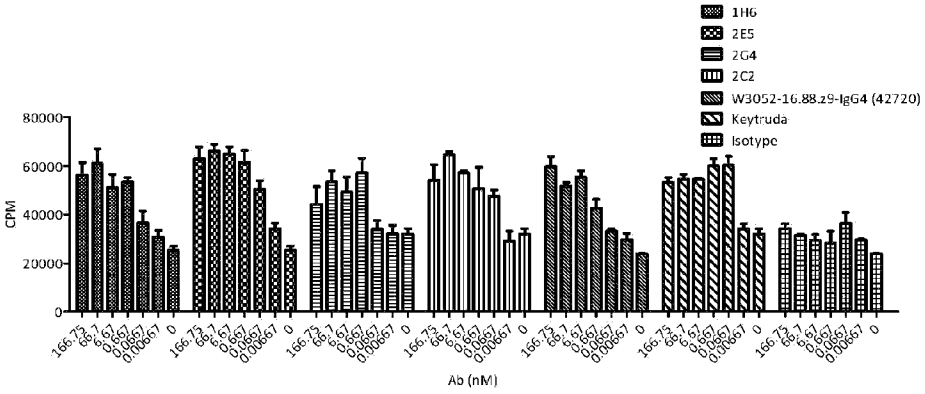
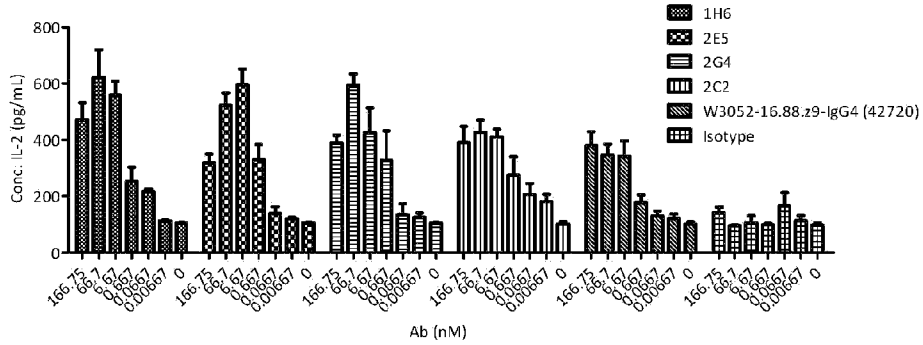


Figure 12

A



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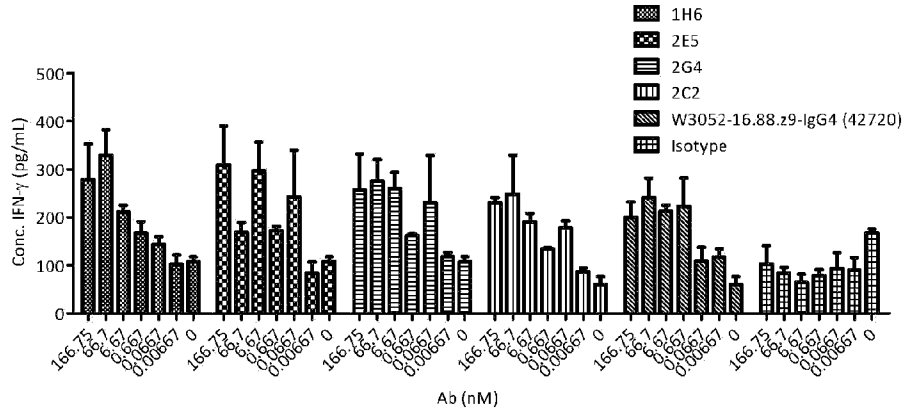

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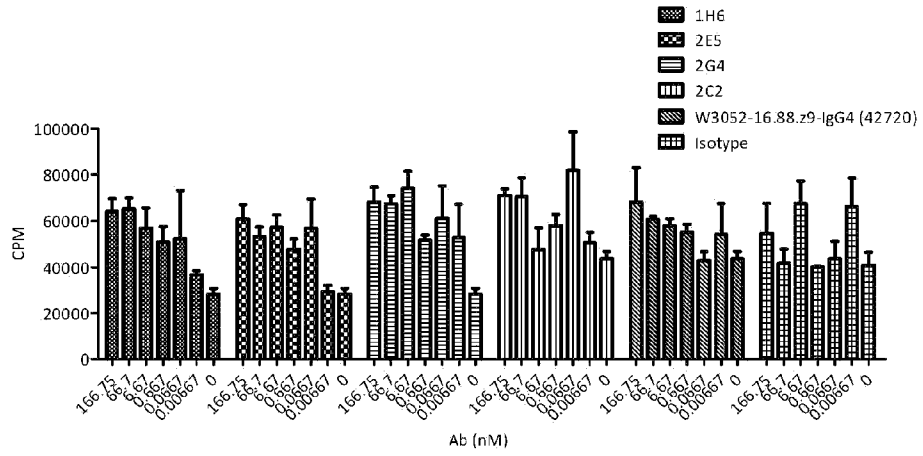
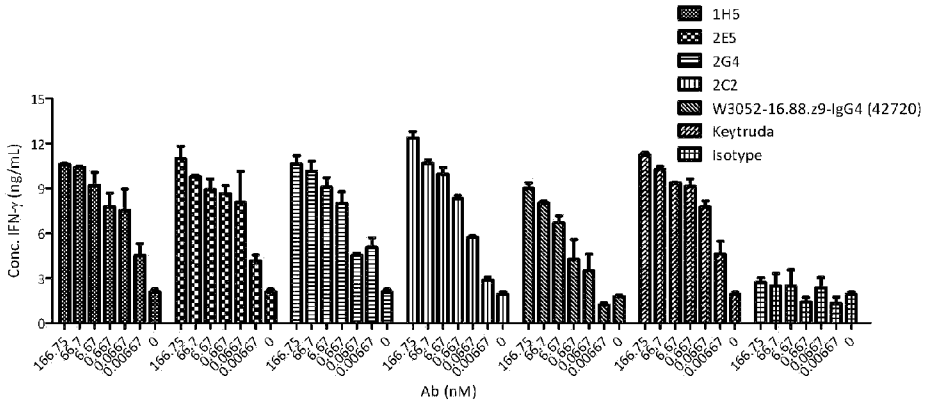


Figure 13

A


1H5
2E5
2G4
2C2
W3052-16.88.z9-IgG4 (42720)
Keytruda
Isotype
Conc. IFN-γ (ng/mL)
Ab (nM)

B

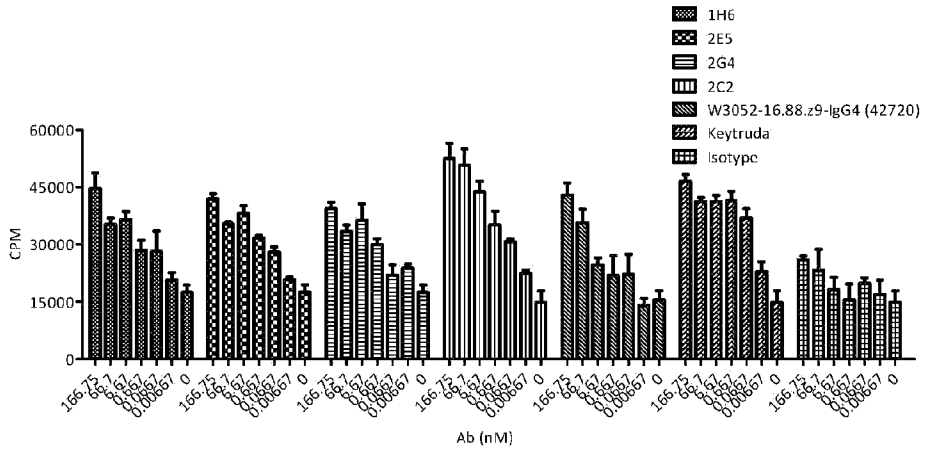
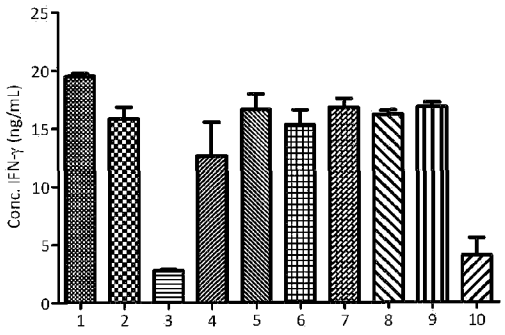

1H6
2E5
2G4
2C2
W3052-16.88.z9-IgG4 (42720)
Keytruda
Isotype
CPM
Ab (nM)

Figure 14

A



NO.	Sample
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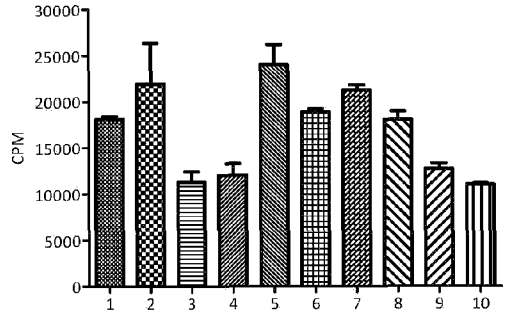
2	Teff(1E5)
3	Teff(1E5)/Treg(1E5)
4	Teff(1E5)/Treg(1E5)/W3052-16.88.z9-IgG4(42720)
5	Teff(1E5)/Treg(1E5)/1H6
6	Teff(1E5)/Treg(1E5)/2C2
7	Teff(1E5)/Treg(1E5)/2E5
8	Teff(1E5)/Treg(1E5)/2G4
9	Teff(1E5)/Treg(1E5)/Keytruda
10	Teff(1E5)/Treg(1E5)/Isotype

B



NO.	Sample
1	Teff(2E5)
2	Teff(1E5)
3	Teff(1E5)/Treg(1E5)
4	Teff(1E5)/Treg(1E5)/W3052-16.88-z9-IgG4(42720)
5	Teff(1E5)/Treg(1E5)/1H6
6	Teff(1E5)/Treg(1E5)/2C2
7	Teff(1E5)/Treg(1E5)/2E5
8	Teff(1E5)/Treg(1E5)/2G4
9	Teff(1E5)/Treg(1E5)/Keytruda
10	Teff(1E5)/Treg(1E5)/Isotype

Figure 15

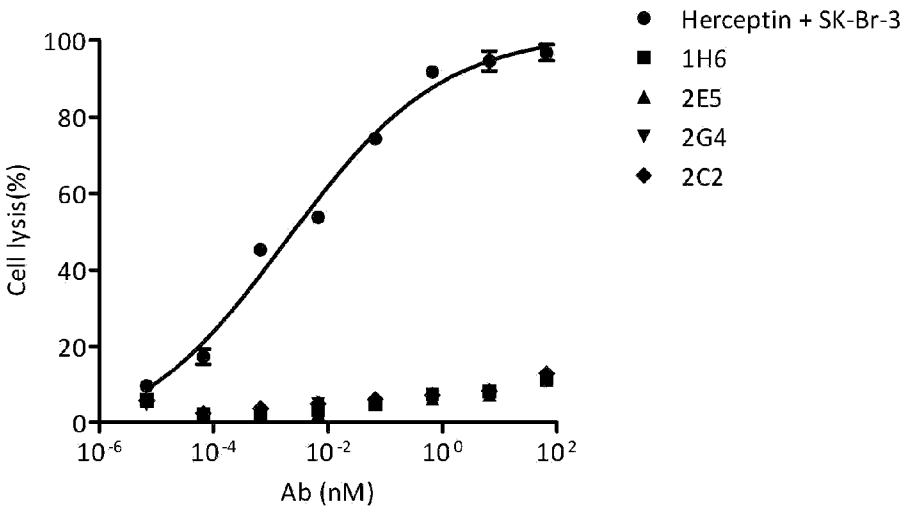


Figure 16

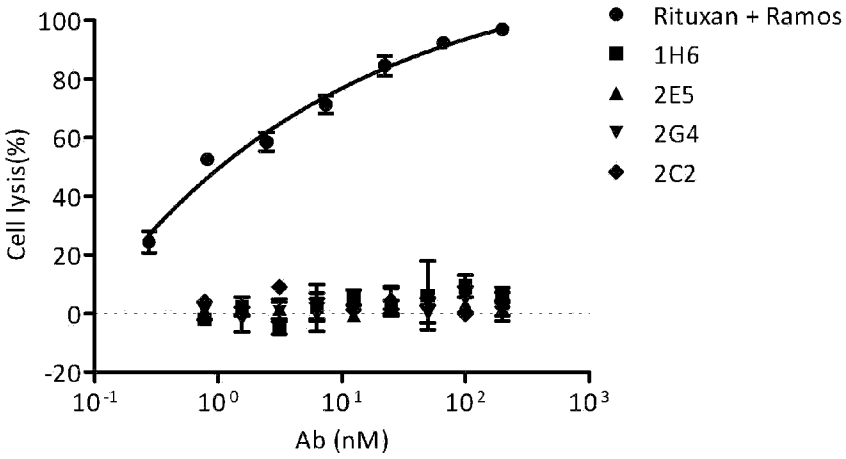


Figure 17

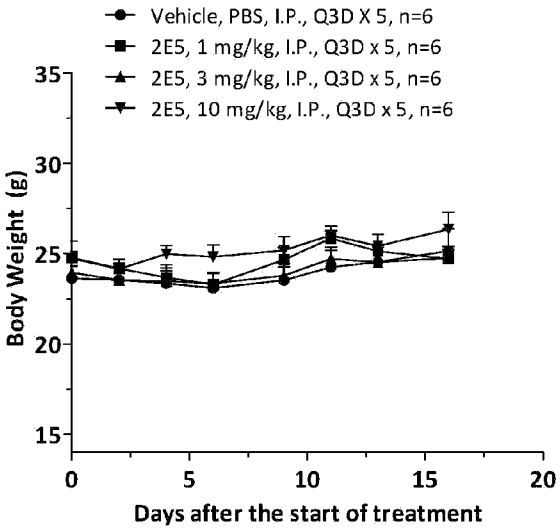


Figure 18

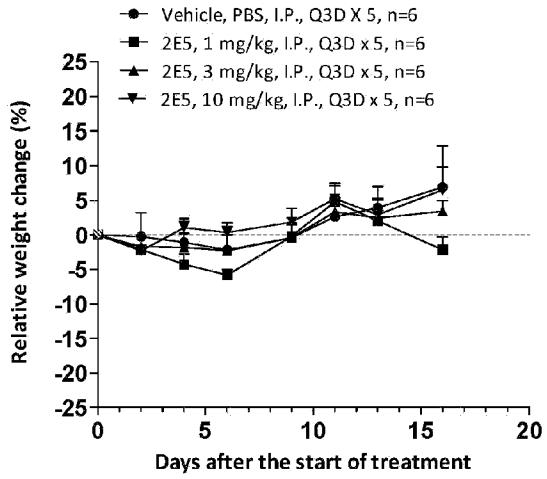


Figure 19

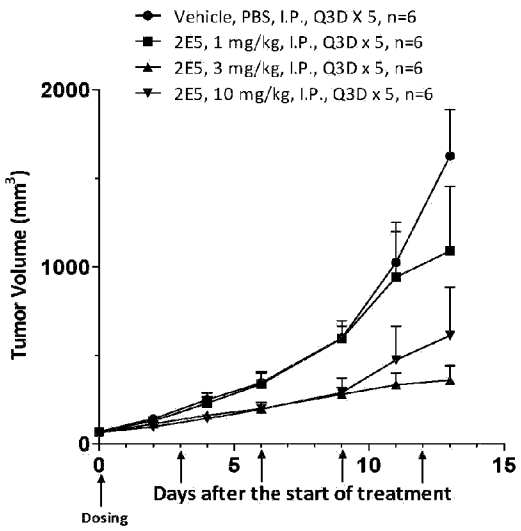


Figure 20

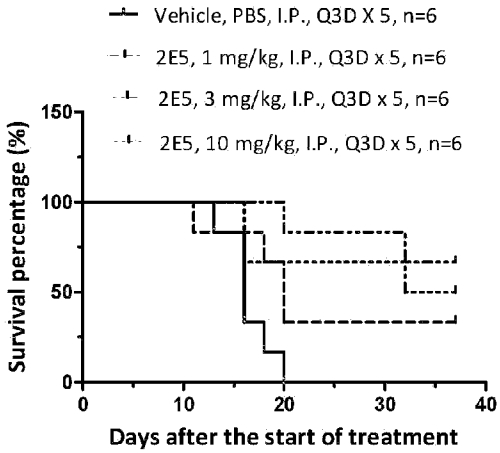


Figure 21

SEKVENSLISTE

Sekvenslisten er udeladt af skriftet og kan hentes fra det Europæiske Patent Register.

The Sequence Listing was omitted from the document and can be downloaded from the European Patent Register.