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(54) **Titre : ANTICORPS ANTI-B7-H4, FRAGMENT DE LIAISON A L'ANTIGENE DE CELUI-CI ET UTILISATION PHARMACEUTIQUE DE CELUI-CI**
(54) **Title: ANTI-B7-H4 ANTIBODY, ANTIGEN-BINDING FRAGMENT THEREOF AND PHARMACEUTICAL USE THEREOF**

(57) **Abrégé/Abstract:**
An anti-B7-H4 antibody, an antigen-binding fragment thereof and pharmaceutical use thereof. A chimeric antibody and a humanized antibody comprising a CDR region of the anti-B7-H4 antibody, a pharmaceutical composition comprising the anti-B7-H4 antibody and the antigen-binding fragment thereof, and use thereof as an anti-cancer medicament. A humanized anti-B7-H4 antibody and use thereof in the preparation of a medicament for treating diseases or conditions mediated by B7-H4.

**Abstract:**

An anti-B7-H4 antibody, an antigen-binding fragment thereof and pharmaceutical use thereof. A chimeric antibody and a humanized antibody comprising a CDR region of the anti-B7-H4 antibody, a pharmaceutical composition comprising the anti-B7-H4 antibody and the antigen-binding fragment thereof, and use thereof as an anti-cancer medicament. A humanized anti-B7-H4 antibody and use thereof in the preparation of a medicament for treating diseases or conditions mediated by B7-H4.



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# ANTI-B7-H4 ANTIBODY, ANTIGEN-BINDING FRAGMENT THEREOF AND PHARMACEUTICAL USE THEREOF

## FIELD OF THE INVENTION

The present invention relates to an anti-B7-H4 antibody, antigen-binding fragment thereof, having immunoreactivity to human B7-H4 receptor, chimeric antibodies and humanized antibodies comprising the CDR regions of said anti-B7-H4 antibody, pharmaceutical compositions comprising the human anti-B7-H4 antibody and antigen-binding fragment thereof, and use thereof as anticancer agents.

## BACKGROUND OF THE INVENTION

Tumor immunotherapy is a long-term research and development hotspot in the field of cancer treatment, and among which T cell tumor immunotherapy is in a central position. Tumor escape is a huge obstacle faced by tumor immunotherapy. Most expressing tumors can be recognized to varying degrees by the host immune system, but in many cases, tumor growth is promoted by the inhibition of the immune system caused by tumor cells per se, due to inadequate immune response triggered by inefficient activation of effector T cells. Tumor immunotherapy is to fully utilize and recruit killer T cells and/or other immune cells in tumor patients to kill tumors.

Studies on the CD28 receptor and its ligands have led to the characterization of related molecules known as the B7 superfamily. Members of the B7 family are a class of immunoglobulins with immunoglobulin V-like domain (IgV) and immunoglobulin C-like domain (IgC), members of which include costimulatory factors B7.1 (CD80) and B7.2 (CD86), inducible ligand for stimulatory factor (ICOS-L/B7-H2), programmed death-1 ligand (PD-L1/B7-H1), programmed death-2 ligand (PD-L2/B7-DC), B7-H4 and B7-H4, etc.

Human B7-H4 is a type I transmembrane protein consisting of 282 amino acids, the coding gene of which is located in the p11.1 region of chromosome 1 (Choi IH et al., J Immunol. 2003 Nov 1; 171(9): 4650-4). B7-H4 plays a role in the negatively regulating T cell immune response. B7-H4 has extensively inhibitory effects on the differentiation, development, cell cycle progression and cytokine production of $CD4^+$ and $CD8^+$ T cells (Sica GL et al. Immunity. 2003 Jun; 18(6): 849-61). Immune cell disorders and autoimmune phenomena were not found in B7-H4 knockout mice (Zhu G et al, Blood. 2009 Feb 19; 113(8): 1759-67; Suh WK et al., Blood. Mol Cell Biol. 2006 Sep; 26(17):6403-11). B7-H4 receptors and their signaling pathways are still unclear now.

Recent studies have found that B7-H4 protein is abundantly expressed in various tumor tissues, allowing tumor cells to escape from the attack of the body's immune system.



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As a target for tumor therapy, B7-H4 molecule provides a new method for tumor immunotherapy.

Currently, it is known that human B7-H4 is expressed on various cancer cells such as breast cancer, ovarian cancer, lung cancer, cervical cancer, kidney cancer, bladder cancer and liver cancer. B7-H4 mRNA expression was found in spleen, lung, thymus, liver, skeletal muscle, kidney, pancreas, testis and ovary. Low expression of B7-H4 at the protein level was found in tissues such as the breast (catheter and lobular), fallopian tube epithelium and endometrial gland. Related studies have also shown that B7-H4 is overexpressed in tumor-associated macrophages (TAM) (Kryczek, I. et al., J. Exp. Med. 2006, 203(4): 871-881), while macrophages constitute an important component of the tumor microenvironment and may account for up to 50% of tumor mass.

At present, numerous national pharmaceutical corporations are engaging in developing monoclonal antibodies against B7-H4 and/or drug-conjugates thereof to improve the patient's own immune system response to tumors and achieve direct killing of tumor cells. Related patents are, for example, WO2013025779, US20140322129 and the like. Anti-B7-H4 monoclonal antibodies available from companies such as Medimmune and FivePrime are currently being at pre-clinical phase; Genentech's anti-B7-H4 antibody-drug conjugates are also at preclinical development phase.

The present invention provides anti-B7-H4 antibodies with high affinity, high selectivity and high biological activity, for use in monoclonal antibody immunotherapy for tumors and related applications thereof. Medicaments, compositions, and methods for the treatment of B7-H4 positive tumors are also provided.

## SUMMARY OF THE INVENTION

The present invention provides a B7-H4 antibody or antigen-binding fragment thereof, comprising:

antibody light chain variable region comprising at least one LCDR selected from the group consisting of:

SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8;

SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16;

SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24;

SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32;

SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40;

SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48;

SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56;

SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64;

SEQ ID NO: 70, SEQ ID NO: 71, SEQ ID NO: 72; and

antibody heavy chain variable region comprising at least one HCDR selected from the group consisting of:



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SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5;
SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13;
SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21;
SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29;
SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37;
SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45;
SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53;
SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61;
SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69;
SEQ ID NO:73, SEQ ID NO:74.

In a preferred embodiment of the invention, an anti-B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein said antibody heavy chain variable region comprises:

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 19, SEQ ID NO: 20 and SEQ ID NO: 21, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 43, SEQ ID NO: 44 and SEQ ID NO: 45, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 51, SEQ ID NO: 52 and SEQ ID NO: 53, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 59, SEQ ID NO: 60 and SEQ ID NO: 61, respectively;

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 67, SEQ ID NO: 68 and SEQ ID NO: 69, respectively; or

HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:73, SEQ ID NO:74 and SEQ ID NO:21, respectively.

In a preferred embodiment of the invention, an anti-B7-H4 antibody or antigen-binding fragment thereof is provided, wherein said antibody light chain variable region comprises:

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 14, SEQ ID NO: 15 and



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SEQ ID NO: 16, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 46, SEQ ID NO: 47 and SEQ ID NO: 48, respectively;

LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 54, SEQ ID NO: 55 and SEQ ID NO: 56, respectively;

LCDR1, LCDR2, and LCDR3 as shown in SEQ ID NO: 62, SEQ ID NO: 63, and SEQ ID NO: 64, respectively; or

LCDR1, LCDR2, and LCDR3 as shown in SEQ ID NO: 70, SEQ ID NO: 71, and SEQ ID NO: 72, respectively.

A particularly preferred anti-B7-H4 antibody or antigen-binding fragment thereof may be any one selected from the group consisting of the followings, comprising one or more following CDR region sequence(s) or sequence(s) showing at least 95% sequence identity thereto:

(1) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, respectively;

(2) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively;

(3) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 19, SEQ ID NO: 20 and SEQ ID NO:21, respectively;

(4) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29, respectively;

(5)the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37, respectively;



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(6) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 46, SEQ ID NO: 47 and SEQ ID NO: 48, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 43, SEQ ID NO: 44 and SEQ ID NO: 45, respectively;

(7) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 54, SEQ ID NO: 55 and SEQ ID NO: 56, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 51, SEQ ID NO: 52 and SEQ ID NO: 53, respectively;

(8) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 62, SEQ ID NO: 63 and SEQ ID NO: 64, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:59, SEQ ID NO:60 and SEQ ID NO:61, respectively;

(9) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:67, SEQ ID NO:68 and SEQ ID NO:69, respectively; and

(10) the antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 shown in SEQ ID NO:73, SEQ ID NO:74 and SEQ ID NO:21, respectively.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the antibody or antigen-binding fragment thereof is a murine antibody or fragment thereof.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof is provided, wherein the antibody or antigen-binding fragment thereof is a chimeric antibody or fragment thereof.

In a preferred embodiment of the invention, an anti-B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the antibody or antigen-binding fragment thereof is a human antibody or fragment thereof.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the antibody or antigen-binding fragment thereof is a humanized antibody or fragment thereof.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody light chain variable region is light chain variable region comprising the sequence(s) selected from the group consisting of SEQ ID NO:76, SEQ ID NO:78, SEQ ID NO:80 or SEQ ID NO:82.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized



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antibody heavy chain variable region is heavy chain variable region comprising the sequence(s) selected from the group consisting of SEQ ID NO:75, SEQ ID NO:77, SEQ ID NO:79 or SEQ ID NO:81.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody heavy chain variable region further comprises heavy chain FR region(s) of human IgG1, IgG2, IgG3 or IgG4 or a variant thereof, preferably comprises heavy chain FR region(s) of human IgG1, IgG2 or IgG4; more preferably comprises heavy chain FR region(s) of IgG1 which have been subjected to amino acid mutation to enhance ADCC toxicity.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody light chain is light chain comprising the sequence selected from the group consisting of SEQ ID NO: 84, SEQ ID NO:86, SEQ ID NO:88 or SEQ ID NO:90.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody heavy chain is heavy chain comprising the sequence selected from the group consisting of SEQ ID NO: 83 SEQ ID NO:85, SEQ ID NO:87 or SEQ ID NO:89.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody light chain variable region is light chain variable region comprising the sequence selected from the group consisting of SEQ ID NO: 76 or SEQ ID NO: 80.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody heavy chain variable region is heavy chain variable region comprising the sequence selected from the group consisting of SEQ ID NO: 75 or SEQ ID NO:79

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody light chain comprising the sequence selected from the group consisting of SEQ ID NO: 84 or SEQ ID NO:88.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the humanized antibody heavy chain comprising the sequence selected from the group consisting of SEQ ID NO: 83 or SEQ ID NO:87.

In a more preferred embodiment of the invention, the humanized antibody is selected from any of the following antibodies comprising:

(1) light chain variable region of SEQ ID NO: 76 and heavy chain variable region of SEQ ID NO: 75;

(2) light chain variable region of SEQ ID NO: 78 and a heavy chain variable



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region of SEQ ID NO: 77;

(3) light chain variable region of SEQ ID NO: 80 and heavy chain variable region of SEQ ID NO: 79; or

(4) light chain variable region of SEQ ID NO: 82 and heavy chain variable region of SEQ ID NO: 81.

In a further preferred embodiment of the invention, the humanized antibody is selected from any one of the following antibodies comprising:

(1) light chain of SEQ ID NO: 84 and heavy chain of SEQ ID NO: 83;

(2) light chain of SEQ ID NO: 86 and heavy chain of SEQ ID NO: 85;

(3) light chain of SEQ ID NO: 88 and heavy chain of SEQ ID NO: 87; or

(4) light chain of SEQ ID NO: 90 and heavy chain of SEQ ID NO:89.

An anti-B7-H4 antibody or antigen-binding fragment thereof, having at least one of the following characteristics: (1) binding to an epitope comprising amino acids 41-60 in SEQ ID NO: 92 of B7-H4; and (2) binding to an epitope comprising amino acids 53-59 in SEQ ID NO: 92 of B7-H4.

An anti-B7-H4 antibody or antigen-binding fragment thereof, having at least one of the following characteristics: (1) binding to an epitope comprising amino acid 53 in SEQ ID NO: 92 of B7-H4; (2) binding to an epitope comprising amino acid 54 in SEQ ID NO: 92 of B7-H4; (3) binding to an epitope comprising amino acid 56 in SEQ ID NO: 92 of B7-H4; (4) binding to an epitope comprising amino acid 57 in SEQ ID NO: 92 of B7-H4; (5) binding to an epitope comprising amino acid 58 in SEQ ID NO: 92 of B7-H4; and (2) binding to an epitope comprising amino acid 59 in SEQ ID NO: 92 of B7-H4.

In a preferred embodiment of the invention, a B7-H4 antibody or antigen-binding fragment thereof as described above is provided, wherein the antigen-binding fragment is Fab, Fv, sFv, $F(ab')_2$, linear antibody, single-chain antibody, nanobody, domain antibody, or multispecific antibody.

The present invention further provides a DNA sequence encoding the B7-H4 antibody or antigen-binding fragment thereof as described above.

The present invention further provides an expression vector comprising the DNA sequence as described above.

The invention further provides a host cell being introduced with or comprising the expression vector as described above.

In a preferred embodiment of the invention, the host cell as described above is characterized in that the host cell is bacterium, preferably *Escherichia coli.*

In a preferred embodiment of the invention, the host cell as described above is yeast, preferably *Pichia pastoris.*

In a preferred embodiment of the invention, the host cell as described above is mammalian cell, preferably Chinese hamster ovary (CHO) cell or human embryonic kidney (HEK) 293 cell.



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The invention also provides a method of producing the B7-H4 antibody, including culturing the host cell as described above, isolating the antibody from the culture, and purifying the antibody.

The invention also provides a multispecific antibody comprising light chain variable region and heavy chain variable region as described above.

The invention also provides a single chain antibody comprising light chain variable region and heavy chain variable region as described above.

The invention also provides a detection reagent or diagnostic agent comprising the B7-H4 antibody or antigen-binding fragment thereof as described above.

The present invention also provides a method for immunodetection or determination of B7-H4, which comprises using the B7-H4 antibody or antigen-binding fragment thereof of the present invention.

The present invention also provides a method for diagnosing diseases associated with B7-H4 positive cells, the method comprises detecting or measuring B7-H4 or B7-H4 positive cells by using the B7-H4 antibody or antigen-binding fragment thereof according to the present invention.

The invention further provides a pharmaceutical composition comprising the B7-H4 antibody or antigen-binding fragment thereof as described above and a pharmaceutically acceptable excipient, dilution or carrier.

The present invention further provides use of the anti-B7-H4 antibody or antigen-binding fragment thereof as described above in the manufacture of a medicament for the treatment of B7-H4 mediated disease or condition; wherein the disease is preferably a cancer; preferably, the disease is B7-H4 expressing cancer; the cancer is most preferably selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, kidney cancer, lung cancer, liver cancer, stomach cancer, colon cancer, bladder cancer, esophageal cancer, cervical cancer, gallbladder cancer, glioblastoma and melanoma.

The present invention further provides a method of treating and preventing B7-H4 mediated diseases or conditions, including administering to a subject in need thereof a therapeutically effective amount of the anti-B7-H4 antibody or antigen-binding fragment thereof, or a pharmaceutical composition comprising the same, wherein the disease is preferably a cancer; preferably, the disease is B7-H4 expressing cancer; the cancer is most preferably selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, kidney cancer, lung cancer, liver cancer, stomach cancer, colon cancer, bladder cancer, esophageal cancer, gallbladder cancer, cervical cancer, glioblastoma and melanoma.



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## DESCRIPTION OF THE DRAWINGS

Figure 1: ELISA binding assay *in vitro* of antibodies, showing that all seven chimeric antibodies have binding activity to the purified human B7-H4 antigen, wherein the chimeric antibodies 2F7 and 2F8 have $EC_{50}$ of about 0.1 nM.

Figure 2: Indirect ELISA binding assays, showing the antigenic epitope to which the anti-B7-H4 antibody hu2F7 binds.

Figure 3: Pharmacological assay in mice, showing the anti-tumor effect of anti-B7-H4 antibody hu1C9.

Figure 4: Immunological function assay, showing effect of anti-B7-H4 antibodies hu1C9 and hu2G6 in enhancing immunity (T cell proliferation).

## DETAILED DESCRIPTION OF THE DISCLOSURE

1. Terminology

In order to more readily understood the invention, certain technical and scientific terms are specifically defined below. Unless specifically defined elsewhere in this document, all other technical and scientific terms used herein have the meaning commonly understood by one of ordinary skill in the art to which this invention belongs.

As used herein, the single-letter code and the three-letter code for amino acids are as described in J. biol. chem, 243, (1968) p3558.

As used herein, the term "antibody" refers to immunoglobulin, a four-peptide chain structure formed by connecting two identical heavy chains and two identical light chains together by interchain disulfide bond(s). Different immunoglobulin heavy chain constant regions exhibit different amino acid compositions and sequences, thereby presenting different antigenicity. Accordingly, immunoglobulins can be divided into five categories, or called immunoglobulin isotypes, namely IgM, IgD, IgG, IgA and IgE, their heavy chains are μ chain, δ chain, γ chain, α chain and ε chain, respectively. According to its amino acid composition of hinge region and the number and location of heavy chain disulfide bonds, the same type of Ig can be divided into different sub-categories, for example, IgG can be divided into IgG1, IgG2, IgG3, and IgG4. Light chain can be divided into κ or λ chain according to different constant regions. Each of the five Igs can have κ or λ chain.

In the present invention, the antibody light chain variable region described herein further comprises a light chain constant region, which comprises a human or murine κ, λ chain or a variant thereof.

In the present invention, the antibody heavy chain variable region described herein further comprises a heavy chain constant region, which comprises human or murine IgG1, 2, 3, 4 or a variant thereof.

The sequences of about 110 amino acids located near the N-terminal of the



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antibody heavy chains and light chains, vary largely, this region is known as variable region (V region); the rest of the amino acid sequence near the C-terminus is relative stable, known as constant region (C region).Variable region comprises three hypervariable regions (HVR) and four relatively conserved sequence framework region (FR). The three hypervariable regions determine the specificity of the antibody, also known as complementarity determining region (CDR). Each light chain variable region (LCVR) and each heavy chain variable region (HCVR) are consisted of three CDRs and four FRs, from the amino terminal to the carboxyl terminal being: FR1, CDR1, FR2, CDR2, FR3, CDR3, and FR4. Three light chain CDRs refer to LCDR1, LCDR2, and LCDR3; three heavy chain CDRs refer to HCDR1, HCDR2 and HCDR3. The numbers and locations of CDR amino acid residues in VL and VH of the antibody or antigen-binding fragment herein comply with the known Kabat numbering criteria and Kabat or AbM definition criteria (http://bioinf.org.uk/abs/).

The term "antigen presenting cell" or "APC" is a cell that displays on its surface foreign antigens complexed with MHC. T cells recognize such complex by T cell receptors (TCRs). Examples of APCs include, but are not limited to, dendritic cells (DC), peripheral blood mononuclear cells (PBMCs), monocytes, B lymphoblasts and monocyte-derived dendritic cells (DC). The term "antigen presentation" refers to a process during which APCs capture antigens and allow them to be recognized by T cells, for example as a component of MHC-I/MHC-II conjugates.

The term "B7-H4" refers to a member of the human B7 protein family, also known as CD276, which is a type I transmembrane protein having four Ig-like extracellular domains. B7-H4 is one of the immune checkpoint proteins expressed on the surface of antigen-presenting cells or cancer cells, and it has an inhibitory effect on activation of T cells. The term "B7-H4" includes any variant or isoform of B7-H4 naturally expressed by cells. The antibodies of the present invention can cross-react with B7-H4 obtained from non-human species. Alternatively, the antibodies may be specific for human B7-H4, and may not exhibit cross-reactivity with other species. B7-H4 or any variant or isotype thereof can be isolated from cells or tissues in which they are naturally expressed, or produced by recombinant techniques using techniques commonly used in the art and those described herein. Preferably, the anti-B7-H4 antibodies target human B7-H4 with normal glycosylation pattern.

The term "recombinant human antibody" includes human antibodies prepared, expressed, created or isolated by recombinant methods, and the techniques and methods involved are well known in the art, such as: (1) an antibody isolated from a human immunoglobulin gene transgenic or transchromosomal animal (e.g., a mouse), or a prepared hybridoma ; (2) an antibody isolated from transformed host cells expressing the antibody, such as a transfectoma; (3) an antibody isolated from a recombinant combinatorial human antibody library; and (4) an antibody prepared, expressed, created or



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isolated by splicing human immunoglobulin gene sequences onto other DNA sequences or the like. Such recombinant human antibody comprises variable region and constant region by incorporating specific human germline immunoglobulin sequences encoded by germline genes, but also subsequent rearrangements and mutations such as those occurred during the antibody maturation.

The term "murine antibody" in the present invention refers to anti-human B7-H4 monoclonal antibody prepared according to the knowledge and skills in the art. During the preparation, a test object is injected with B7-H4 antigen, and then hybridoma expressing antibody which possesses desired sequence or functional characteristics is isolated. In a preferred embodiment of the present application, the murine B7-H4 antibody or the antigen-binding fragment thereof further comprises a light chain constant region of murine κ, λ chain or a variant thereof, or further comprises a heavy chain constant region of murine IgG1, IgG2, IgG3 or IgG4, or a variant thereof.

The term "human antibody" includes antibodies having variable and constant regions from human germline immunoglobulin sequences. Human antibodies of the present invention may include amino acid residues that are not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*). However, the term "human antibody" does not include an antibody in which CDR sequences derived from other mammalian species germline, such as mouse germline, have been grafted onto a human framework sequence (i.e., "humanized antibody").

The term "humanized antibody", also known as CDR-grafted antibody, refers to an antibody generated by grafting murine CDR sequences into a variable region framework of human antibody. Humanized antibodies avoid the undesired strong antibody response induced by the chimeric antibodies which carry a large amount of murine protein components. To avoid a decrease in the activity caused by the reduced immunogenicity, the variable region of the human antibody can be subjected to a minimum back mutation to maintain the activity.

The term "chimeric antibody", is an antibody which is formed by fusing the variable region of a murine antibody with the constant region of a human antibody, and the chimeric antibody can alleviate the murine antibody-induced immune response. To establish a chimeric antibody, hybridoma secreting specific murine monoclonal antibody is firstly established, a variable region gene is cloned from mouse hybridoma cells, then a constant region gene of a human antibody is cloned as desired, the mouse variable region gene is ligated with the human constant region gene to form a chimeric gene which then can be inserted into a human vector, and finally the chimeric antibody molecule is expressed in eukaryotic or prokaryotic industrial system. The constant region of a human antibody is selected from the heavy chain constant region of human IgG1, IgG2, IgG3 or IgG4 or a variant thereof, preferably is the heavy chain constant region of human IgG2 or



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IgG4, or the heavy chain constant region of IgG1 which exhibits increased ADCC (antibody-dependent cell-mediated cytotoxicity), due to amino acid mutation.

The term "antigen-binding fragment" refers to antigen-binding fragments of an antibody and analogs of an antibody, which generally include at least a part of the antigen-binding region or variable region (e.g., one or more CDRs) of the parental antibody. Antibody fragments retain at least some of the binding specificity of the parent antibody. Generally, when the activity is expressed as molar, antibody fragments retain at least 10% binding activity of the parental antibody. Preferably, the antibody fragments retain at least 20%, 50%, 70%, 80%, 90%, 95%, or 100% or more binding affinity of the parental antibody to the target. Examples of antigen-binding fragments include, but are not limited to Fab, Fab', F(ab')2, Fv fragments, linear antibodies, single chain antibodies, nanobodies, domain antibodies, and multispecific antibodies. Engineered antibody variants are reviewed in Holliger and Hudson (2005) Nat. Biotechnol. 23: 1126-1136.

"Fab fragment" is composed of a light chain, a heavy chain CH1 and variable regions. The heavy chain of a Fab molecule cannot form disulfide bond with another heavy chain molecule.

"Fc" region contains two heavy chain fragments comprising the antibody CH1 and CH2 domains. The two heavy chain fragments are held together by two or more disulfide bonds and hydrophobic interaction of the CH3 domain.

"Fab' fragment" contains a light chain and part of a heavy chain comprising VH domain, CH1 domain and a region between the CH1 and CH2 domains, and thereby a F(ab')2 molecule can be formed by two heavy chains of two Fab' fragments linked by interchain disulfide bonds.

"F(ab')2 fragment" contains two light chains and part of heavy chains comprising the constant region between CH1 and CH2 domains, thereby interchain disulfide bonds are formed between the two heavy chains. Therefore, the F(ab')2 fragment consists of two Fab' fragments held together by disulfide bonds between the two heavy chains.

"Fv region" contains variable regions from both heavy and light chains, but without constant regions.

The term "multispecific antibody" is used in its broadest sense and encompasses antibodies with multi-epitope specificity. These multispecific antibodies include, but are not limited to antibodies comprising a heavy chain variable region (VH) and a light chain variable region (VL), where the VH-VL unit has multiple epitope specificity; antibodies comprising two or more VL and VH regions, where each VH-VL unit binds to different targets or to different epitopes of the same target; antibodies comprising two or more single variable regions, where each single variable region binds to different targets or to different epitopes of the same target; full-length antibodies; antibody fragments; diabodies; bispecific diabodies and triabodies, antibody fragments which have been covalently or



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non-covalently linked together.

The term "single-chain antibody" is a single-chain recombinant protein composed of antibody heavy chain variable region (VH) and light chain variable region (VL) connected by a peptide linker, and the single-chain antibody is the smallest antibody fragment with intact antigen binding sites.

The term "domain antibody fragment" is an immunoglobulin fragment having immunological functions and it only contains heavy chain variable region or light chain variable region chain. In some cases, two or more VH regions are covalently linked to a peptide linker to form a bivalent domain antibody fragment. The two VH regions of a bivalent domain antibody fragment can target the same or different antigens.

The term "binding to B7-H4", refers to the interaction with human B7-H4. The term "antigen binding site" as used herein refers to discontinuous three-dimensional sites on the antigen, recognized the antibody or the antigen-binding fragment of the present application.

The term "epitope" refers to the sites on an antigen that specifically bind to an immunoglobulin or antibody. The epitope can be formed by adjacent amino acids, or by non-adjacent amino acids but brought to be closed due to tertiary folding of a protein. The epitope formed by adjacent amino acids is typically retained after exposure to denaturing solvents, whereas the epitope formed by tertiary folding is typically lost after treatment with denaturing solvents. Epitopes typically include at least 3-15 amino acids in a unique spatial conformation. Methods for determining what epitope is bound by a given antibody are well known in the art, including immunoblotting and immunoprecipitation assays, and the like. Methods for determining the spatial conformation of an epitope include techniques in the art and techniques described herein, such as X-ray crystallography and two-dimensional nuclear magnetic resonance.

The term "specifically binds to", "selectively binds to" as used herein, refers to the binding of an antibody to an epitope on a predetermined antigen. Typically, where a recombinant human B7-H4 is used as analyte and an antibody is used as ligand, the antibody binds to a predetermined antigen at approximately less than $10^{-7}$ M or even less equilibrium dissociation constant ($K_D$), and the affinity of the antibody for binding to the predetermined antigen is at least two times higher than that for non-specific antigens (other than the predetermined antigen or closely related antigens) (such as BSA), as measured in an instrument via surface plasmon resonance (SPR) techniques. The term "an antibody which recognizes the antigen" can be used interchangeably herein with the term "specifically binding antibody".

The term "cross reaction" refers to the ability of the antibody of the present invention to bind to B7-H4 derived from different species. For example, an antibody of the present invention that binds to human B7-H4 can also bind to B7-H4 derived from another species. Cross-reactivity is measured by detecting the specific reactivity with purified



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antigen in binding assays (e.g., SPR and ELISA), or by detecting the binding or functional interaction with cells physiologically expressing B7-H4. Methods for determining cross-reactivity include standard binding assays as described herein, such as surface plasmon resonance (SPR) analysis, or flow cytometry.

The terms "inhibition" or "blockade" are used interchangeably and encompass both partial and complete inhibition/blockade. Inhibition/blockade of ligand preferably reduces the normal ligand-binding level or alters type of ligand-binding activity, when compared with that in the absence of inhibition or blockade. Inhibition and blockade are also intended to include any measurable decrease in binding affinity where a ligand is contacted with an anti-B7-H4 antibody, when compared with the binding affinity in the absence of anti-B7-H4 antibody.

The term "inhibiting the growth" (e.g., in the context of cells) is intended to include any measurable reduction in cell growth.

The terms "inducing an immune response" and "enhancing an immune response" are used interchangeably and refer to the immune response to the stimulation of a particular antigen (i.e., passive or adaptive). With respect to the induction of CDC or ADCC, the term "inducing" means to stimulate a specific mechanism to directly kill cells.

As used herein, the term "ADCC", namely antibody-dependent cell-mediated cytotoxicity, refers to cells expressing Fc receptors directly kill target cells coated by an antibody through recognizing the Fc segment of the antibody. ADCC effector function of the antibody can be reduced or eliminated via modification of the Fc segment of IgG. The modification refers to mutation(s) of the antibody heavy chain constant region.

Methods for producing and purifying antibodies and antigen-binding fragments are well known in the art and can be found, for example, in Using Antibodies A Laboratory Manual Cold Spring Harbor, Chapter 5-8 and 15. For example, mice can be immunized with human B7-H4, or fragments thereof, and the resulting antibodies can then be renatured, purified and sequenced using conventional methods well known in the art. Antigen-binding fragments can also be prepared by conventional methods. The antibody or the antigen-binding fragment of the present invention can be obtained by introducing one or more human framework regions (FRs) into non-human derived CDRs using genetic engineering. Human FR germline sequences can be obtained from ImMunoGeneTics (IMGT) on website http://imgt.cines.fr, or from The Immunoglobulin FactsBook, 2001ISBN012441351.

The engineered antibody or antigen-binding fragment of the present invention may be prepared and purified using conventional methods. For example, cDNA sequences encoding corresponding antibodies may be cloned and recombined into a GS expression vector. The recombined immunoglobulin expression vector may then be stably transfected into CHO cells. As a more recommended method well known in the art, mammalian expression systems may result in glycosylation of antibodies, typically at the highly



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conserved N-terminus in the $F_C$ region. Stable clones may be obtained through expression of an antibody specifically binding to human antigen. Positive clones may be expanded in a serum-free culture medium for antibody production in bioreactors. Culture medium, into which an antibody has been secreted, may be purified and collected by conventional techniques. The antibody may be filtered and concentrated using common techniques. Soluble mixtures and aggregates may be effectively removed by common techniques, such as molecular sieve or ion exchange. The resulting product should be immediately frozen, for example at -70°C, or may be lyophilized.

The antibodies of the present invention refer to monoclonal antibodies. Monoclonal antibody or mAb, as used herein, refers to an antibody that is derived from a single clone including but is not limited to eukaryotic, prokaryotic, or phage single clone strain. Monoclonal antibodies or antigen-binding fragments thereof can be obtained, for example, by hybridoma technologies, recombinant technologies, phage display technologies, synthetic technologies (e.g., CDR-grafting), or other technologies known in the art.

"Administration" and "treatment," as it applies to an animal, human, experimental subject, cell, tissue, organ, or biological fluid, refers to contacting an exogenous pharmaceutical, therapeutic, diagnostic agent, or composition with the animal, human, subject, cell, tissue, organ, or biological fluid. "Administration" and "treatment" can refer, e.g., to therapeutic, pharmacokinetic, diagnostic, research, and experimental methods. Treatment of a cell encompasses contacting a reagent with the cell, as well as contacting a reagent with a fluid, where the fluid is in contact with the cell. "Administration" and "treatment" also mean in vitro and ex vivo treatments, e.g., of a cell, by a reagent, diagnostic, binding composition, or by another cell. "Treatment", as it applies to a human, veterinary, or a research subject, refers to therapeutic treatment, prophylactic or preventative measures, research and diagnostic applications.

"Treat" means to administer a therapeutic agent, such as a composition comprising any of the binding compounds of the present invention, internally or externally to a patient suffering from one or more disease symptoms for which the agent has known therapeutic activity. Typically, the therapeutic agent is administered in an amount effective to alleviate one or more disease symptoms in the treated patient or population, either by inducing the regression of symptom(s) or by inhibiting the progression of such symptom(s) to any clinically measurable degree. The amount of a therapeutic agent that is effective to alleviate any particular disease symptom (also referred to "therapeutically effective amount") may vary according to factors such as the disease state, age, and weight of the patient, and the ability of the agent to elicit a desired response in the patient. Whether a disease symptom has been alleviated can be assessed by any clinical measurement typically used by physicians or other skilled healthcare providers to assess the severity or progression status of that symptom. While an embodiment (e.g., a treatment method or



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article of manufacture) of the present invention may not be effective in alleviating the target disease symptom(s) of interest in each patient, it should alleviate the target disease symptom(s) of interest in a statistically significant number of patients as determined by any statistical test known in the art such as the Student's t-test, chi-square test, U-test according to Mann and Whitney, Kruskal-Wallis test (H-test), Jonckheere-Terpstra-test and the Wilcoxon-test.

The term "consisting essentially of" or variations thereof used throughout the specification and claims means to involve all the elements or groups of elements, and optionally other elements showing similar property or even different property from that of said elements. Said other elements do not significantly change the substantial or novel property of the given dosing regimen, method, or composition. As a non-limiting example, a binding compound consisting essentially of the described amino acid sequence may also include one or more amino acid(s), which do not significantly affect the properties of the binding compound.

The term "naturally occurring" as applied to an object in the present invention refers to the fact that the object can be found in nature. For example, polypeptide sequences or polynucleotide sequences that exist in organisms (including viruses) and have not been artificially modified in the laboratory are naturally occurring, wherein said organism can be isolated from natural sources.

"Effective amount" encompasses an amount sufficiently to ameliorate or prevent a symptom or sign of a medical condition. Effective amount also means an amount sufficiently to allow or facilitate diagnosis. An effective amount for a particular patient or veterinary subject may vary depending on factors such as the condition being treated, the general health of the patient, the route and dose of administration and the severity of side effects. An effective amount can be the maximal dose or dosing protocol that avoids significant side effects or toxic effects.

"Exogenous" refers to substances that are produced outside an organism, cell, or human body, depending on the context. "Endogenous" refers to substances that are produced within a cell, organism, or human body, depending on the context.

"Homology" refers to sequence similarity between two polynucleotide sequences or between two polypeptides. When a position within two sequences to be compared is occupied by the same base or amino acid monomer subunit, e.g., if a position within each of two DNA molecules is occupied by adenine, then the molecules are homologous at this position. The percentage of homology between two sequences is a function, in which the number of matching or homologous positions shared by the two sequences is divided by the number of positions to be compared, and then multiplied by 100. For example, if 6 out of 10 positions in two sequences are matched or homologous when the sequences are optimally aligned, then the two sequences are deemed as 60% homologous. Generally, the comparison is performed, when two sequences are aligned to



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give maximum homology percentage.

As used herein, the expressions "cell," "cell line," and "cell culture" are used interchangeably and all such designations include progeny thereof. Thus, the wordings "transformants" and "transformed cells" include the primary subject cell and cultures derived therefrom without considering the number of passages. It should also be understood that all progenies may not be precisely identical in DNA content, due to intended or unintended mutations. Mutant progenies thus screened that exhibit the same function or biological activity as that of originally transformed cell are also taken into consideration. Where distinct designations are intended, it will be obvious from the context.

"Optional" or "optionally" means that the event or circumstance that follows may but not necessarily happen, and the description includes the instance in which the event or circumstance shall or shall not happen. For example, "optionally comprises 1-3 antibody heavy chain variable regions" means the antibody heavy chain variable region having specific sequence may be, but not necessarily be present.

"Pharmaceutical composition" refers to a mixture comprising one or more compound(s) according to the present invention or a physiologically/pharmaceutically acceptable salt or prodrug thereof, along withother chemical components, as well as additional components such as physiologically/pharmaceutically acceptable carriers and excipients. The pharmaceutical composition aims at promoting the administration to an organism, facilitating the absorption of the active ingredient and thereby exerting a biological effect.

Hereinafter, the present invention is further described with reference to examples; however, the scope of the present invention is not limited thereto. In the examples of the present invention, where specific conditions are not described, the experiments are generally conducted under conventional conditions as described in Antibodies A Laboratory Manual, Molecular Cloning, Cold Spring Harbor, or under conditions proposed by the material or product manufacturers. Where the source of the reagents is not specifically provided, the reagents are commercially available conventional reagents.

**Example 1: Preparation of antigens and construction of stable cell lines**

Sequence encoding Human B7-H4 with HisFlag tag (huB7-H4-HF), and sequence encoding human B7-H4 with huFc tag (h-B7-H4-Fc) were synthesized by CRO Integrated DNA Technology (IDT) (the template sequence for each of the above B7-H4 recombinant proteins was designed by the inventors), and were cloned into pTT5 vector (Biovector) respectively. The B7-H4 recombinant proteins were expressed in 293T cells and purified according to Example 2.

The purified proteins were used in the following examples.



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huB7-H4-Fc sequence:

FGISGRHSITVTTVASAGNIGEDGILSCTFEPDIKLSDIVIQWLKEGVLGLVHEFKEG
KDELSEQDEMFRGRTAVFADQVIVGNASLRLKNVQLTDAGTYKCYIITSKGKGNA
NLEYKTGAFSMPEVNVDYNASSETLRCEAPRWFPQPTVVWASQVDQGANFSEVS
NTSFELNSENVTMKVVSVLYNVTINNTYSCMIENDIAKATGDIKVTESEIKRRSHL
QLLNSKAGSGGGGDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVV
VDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNG
KEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGF
YPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
MHEALHNHYTQKSLSLSPGK

SEQ ID NO: 99

huB7-H4-his sequence:

MASLGQILFWSIISIIIILAGAIALIIGFGISGRHSITVTTVASAGNIGEDGILSCTFEPDI
KLSDIVIQWLKEGVLGLVHEFKEGKDELSEQDEMFRGRTAVFADQVIVGNASLRL
KNVQLTDAGTYKCYIITSKGKGNANLEYKTGAFSMPEVNVDYNASSETLRCEAPR
WFPQPTVVWASQVDQGANFSEVSNTSFELNSENVTMKVVSVLYNVTINNTYSCMI
ENDIAKATGDIKVTESEIKRRSHLQLLNSKADYKDDDDKGSHHHHHHHH

SEQ ID NO: 100

Purification steps for huB7-H4-his:

The supernatant samples expressed by cells were centrifuged at high speed to remove impurities, subjected to buffer exchange with PBS, and added with imidazole to a final concentration of 5 mM. The nickel column was equilibrated with PBS solution comprising 5 mM imidazole and rinsed with 2-5 column volumes. After buffer exchange, the supernatant samples were applied to the column. The column was washed with PBS comprising 5 mM imidazole until the A280 reading returned to baseline. The column was then washed with PBS + 10 mM imidazole, the non-specifically bound impurity proteins were removed, and the effluent was collected. The target protein was eluted with PBS comprising 300 mM imidazole, and the elution peak was collected. The collected eluate was further purified by ion exchange (SP column). Stock Solution A: 0.01 M PB, pH 8.0. Stock Solution B: Solution A + 1 M NaCl. For the target protein elution, the PBS-imidazole solution was replaced with Solution A, and the SP column was equilibrated with solution A. And then, the samples were applied onto the column. The column was then washed with Solution B at a concentration gradient from 0 to 100%, with 10 column volumes, and the elution peak was collected. The resulting protein was identified as desired protein via electrophoresis, and aliquoted for use. Human B7-H4 with the HisFlag tag (hu-B7-H4 his) was obtained.

Purification steps for huB7-H4-Fc:

The supernatant samples expressed by HEK293 cells were centrifuged at high speed to remove impurities, and subjected to buffer exchange with PBS. The Protein A



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affinity column was equilibrated with 10 mM phosphate buffer, and rinsed with 2-5 column volumes. After buffer exchange, the supernatant samples were applied onto the column. The column was rinsed with buffer at 25 column volumes until the A280 reading returned to the baseline. The target protein was eluted with 0.8% acetate buffer, pH 3.5, and the elution peak was collected. The aliquots were immediately added with 1M Tris-Cl buffer, pH 8.0 for neutralization. And then the solution was exchanged with PBS via Millipore's Amico-15 filter column. The resulting protein was identified by electrophoresis, peptide mapping and LC-MS, and aliquoted for use.

Construction of stable CHO-S cell pool:

The full-length sequence encoding human or cynomolgus B7-H4 protein (huB7-H4 or cyB7-H4) was synthesized by Integrated DNA Technology (IDT) (the above B7-H3 recombinant proteins were designed by the present inventors) and was cloned into engineered pcDNA3.1 vector, pcDNA3.1/puro (Invitrogen #V79020), respectively. CHO-S (ATCC) cells were cultured in CD-CHO culture medium (Life Technologies, #10743029) to reach 0.5 x $10^6$/ml. 10 μg of the vector encoding the huB7H3 or cyB7H3 gene was mixed with 50 μl of LF-LTX (Life Technologies, #A12621) in 1 ml Opti-MEM medium (Life Technologies, #31985088), incubated at room temperature for 20 minutes, added with culture medium for CHO cells, and placed in an incubator with $CO_2$ for cultivation. After 24 hours, the medium was changed with fresh medium and 10 μg/ml puromycin was added. After that, the culture medium was changed every 2-3 days, and stable CHO-S cell pool was obtained after 10-12 day by screening.

**Example 2: Obtaining murine hybridomas and antibody sequences**

Animals were immunized with human antigen huB7-H4-Fc. Five Balb/c and five A/J mice (female, 10 weeks old) were used. The immunogen and the immunoadjuvant (Sigma Complete Freund's Adjuvant (CFA) or Sigma Incomplete Freund's Adjuvant (IFA)) were thoroughly mixed at a ratio of 1:1 and emulsified to prepare a stable "water-in-oil" liquid; The dose for injection was 25 μg/200 μL/mouse.

| | |
|---|---|
| Day 1 | first immunization, CFA |
| Day 21 | second immunization, IFA |
| Day 35 | third immunization, IFA |
| Day 42 | Blood collection and detection of serum titer (blood collected after three times of immunization) |
| Day 49 | forth immunization, IFA |
| Day 56 | Blood collection and detection of serum titer (blood collected after four times of immunization) |

Serum titer and the ability to bind to cell surface antigens were evaluated with sera from the immunized mice by indirect ELISA and Capture ELISA method as described



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in Example 3. Cell fusion was initiated depending on the results of the titer assay (greater than 100,000-fold dilution). The mice with strong serum titer, affinity and FACS binding were subjected to a final immunization and then were sacrificed. Spleen cells and SP2/0 myeloma cells were fused and plated onto the plate to obtain hybridomas, which was screened by indirect ELISA and capture ELISA to obtain target hybridomas. Monoclonal cell strains were established by limiting dilution. The resulting positive antibody strains were further transfected into CHO-S cells stably expressing B7-H4. Blank CHO-S cells were used as control to exclude hybridoma strains of non-specific binding antibodies. Eight hybridoma strains which not only bind to recombinant proteins but also bind to antigens expressed by cells were obtained by flow sorting. Hybridoma cells at logarithmic growth phase were collected, RNAs were extracted with Trizol (Invitrogen, 15596-018) and reverse transcribed (PrimeScript™ Reverse Transcriptase, Takara #2680A). The cDNAs obtained by reverse transcription were amplified by PCR amplification using mouse Ig-Primer Set (Novagen, TB326 Rev. B 0503) and sequenced, and finally sequences of 8 murine antibodies were obtained.

The heavy and light chain variable region sequences of murine mAb 2F7 are as follows:

2F7 HCVR

EVQLVESGGGLVQPGGSLKLSCAASGFTFSNYYMSWVRQTPEKRLEWVAYVSSG GGSTYYSDSVKGRFTISRDNAKNTLYLQMSSLKPEDTAMYYCTRESYSQGNYFDY WGQGTTLTVSS

SEQ ID NO: 1

2F7 LCVR

DIVMTQSPATLSVTPGDRVSLSCRASQSISDYLHWYQQKSHESPRLLIKFASQSISGI PSRFSGSGSGSDFTLSINSVEPEDVGVYYCQNGHSFSLTFGAGTKLELK

SEQ ID NO: 2

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GFTFSNYYMS | SEQ ID NO: 3 |
| HCDR2 | YVSSGGGSTYYSDSVKG | SEQ ID NO: 4 |
| HCDR3 | ESYSQGNYFDY | SEQ ID NO: 5 |
| LCDR1 | RASQSISDYLH | SEQ ID NO: 6 |
| LCDR2 | FASQSIS | SEQ ID NO: 7 |
| LCDR3 | QNGHSFSLT | SEQ ID NO: 8 |

The heavy and light chain variable region sequences of M1 are as follows:



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M1 HCVR

EIQLQQSGPELVMPGASVKVSCTASGYPFTTYNMYWVKQSHGKSLEWIAYIDPYN GGTSYNQKFKGKATLTVDKSSSTAYMHLNSLTSEDSAVYYCARSGFYDGYYAWYF DVWGAGTTVTVSS

SEQ ID NO: 9

M1 LCVR

DVVMTQTPLSLPVSLGDQASISCRSSQSLVHSGGNTYLHWYLQKPGQSPKLLIYK VSNRFSGVPDRFSGSGSGTDFTLKISRVEAEDLGVYFCSQSTHVPLTFGAGTKLEL K

SEQ ID NO: 10

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GYPFTTYNMY | SEQ ID NO: 11 |
| HCDR2 | YIDPYNGGTSYNQKFKG | SEQ ID NO: 12 |
| HCDR3 | SGFYDGYYAWYFDV | SEQ ID NO: 13 |
| LCDR1 | RSSQSLVHSGGNTYLH | SEQ ID NO: 14 |
| LCDR2 | KVSNRFS | SEQ ID NO: 15 |
| LCDR3 | SQSTHVPLT | SEQ ID NO: 16 |

The heavy and light chain variable region sequences of murine mAb 2F8 are as follows:

2F8 HCVR

QVQLQQPGSVLVRPGASVKLSCKASGYTFTNSWMNWAKLRPGQGLEWIGGIYPN SGNIEYNEKFKGKATLTVDTSSSTAYMDLTSLTSEDSAVYYCARDSRFSYWGQGTL VTVSA

SEQ ID NO: 17

2F8 LCVR

DIVMTQSHKFMSTSVGDRVSITCKASQDVRTAVAWYQQKPGQSPKLLISSTSYRYT GVPDRFTGSGSGTDFTFIISSVQAEDLAVYYCQQHYSTPLTFGAGTKLELK

SEQ ID NO: 18

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GYTFTNSWMN | SEQ ID NO: 19 |
| HCDR2 | GIYPNSGNIEYNEKFKG | SEQ ID NO: 20 |



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| | | |
|---|---|---|
| HCDR3 | DSRFSY | SEQ ID NO: 21 |
| LCDR1 | KASQDVRTAVA | SEQ ID NO: 22 |
| LCDR2 | STSYRYT | SEQ ID NO: 23 |
| LCDR3 | QQHYSTPLT | SEQ ID NO: 24 |

The heavy and light chain variable region sequences of murine mAb 2F4 are as follows:

2F4 HCVR

EVQLVESGGGLVKPGGSLKLSCAASGLTFSRYAMSWVRQTPEKRLEWVAGISSGG SYTYYSDTVKGRFTISRDNVRNTLYLQMSSLRSEDTAMYYCGREYGRDYWGQGT SVTVSS

SEQ ID NO: 25

2F4 LCVR

DILMTQSPSSMSVSLGDTVSITCHASQGINSNIGWLQQKPGKSFKGLIYHGTNLED GVPSRFSGSGSGTDYSLTISSLESEDFADYYCVQYAQFPRTFGGGTTLEIK

SEQ ID NO: 26

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GLTFSRYAMS | SEQ ID NO: 27 |
| HCDR2 | GISSGGSYTYYSDTVKG | SEQ ID NO: 28 |
| HCDR3 | EYGRDY | SEQ ID NO: 29 |
| LCDR1 | HASQGINSNIG | SEQ ID NO: 30 |
| LCDR2 | HGTNLED | SEQ ID NO: 31 |
| LCDR3 | VQYAQFPRT | SEQ ID NO: 32 |

The heavy and light chain variable region sequences of murine mAb 2A10 are as follows:

2A10 HCVR

EVQLVESGGGFVKPGGSLKLSCAASGFTFSTFGMSWVRQTPDKRLEWVAGISPGG SYTYYPDTVKGRFTISRDNARNTLYLQMSSLRSEDSAMYYCTRGRSVWGTGTTV TVSS

SEQ ID NO: 33

2A10 LCVR

DILMTQSPSSMSVSLGDTVSITCHASQDISSNIGWLQQKPGKSFKGLIYHGTTLEDG



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IPSRFSGSGSGADYSLTISSLESEDFADYYCVQSAQFPWTFGGGTKLEIK

SEQ ID NO: 34

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GFTFSTFGMS | SEQ ID NO: 35 |
| HCDR2 | GISPGGSYTYYPDTVKG | SEQ ID NO: 36 |
| HCDR3 | GRSV | SEQ ID NO: 37 |
| LCDR1 | HASQDISSNIG | SEQ ID NO: 38 |
| LCDR2 | HGTTLED | SEQ ID NO: 39 |
| LCDR3 | VQSAQFPWT | SEQ ID NO: 40 |

The heavy and light chain variable region sequences of murine mAb 2E4 are as follows:

2E4 HCVR

QVQLQQPGSVLVRPGTSVKLSCKASGYTFTSSWMNWVKQRPGQGLEWIGGIYPN RGTTEYNEKFKGKATLTVDTSSSTAFMDLNRLTSEDSAVYYCARDSRFADWGQGT LVTVSA

SEQ ID NO: 41

2E4 LCVR

DIMLTQSHKFMSTSVGDRVSITCKASQDVSAAVAWYQQKPGQSPKLLISSASYRYT GVPDRFTGSGSGTDFTFTISSVQAEDLAVYYCQQHYNTPLTFGAGTKLELK

SEQ ID NO: 42

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GYTFTSSWMN | SEQ ID NO: 43 |
| HCDR2 | GIYPNRGTTEYNEKFKG | SEQ ID NO: 44 |
| HCDR3 | DSRFAD | SEQ ID NO: 45 |
| LCDR1 | KASQDVSAAVA | SEQ ID NO: 46 |
| LCDR2 | SASYRYT | SEQ ID NO: 47 |
| LCDR3 | QQHYNTPLT | SEQ ID NO: 48 |

The heavy and light chain variable region sequences of murine mAb 1E4 are as follows:

1E4 HCVR

EVQLVESGGGLVKPGGSLKLSCAASGFTFSRYAMSWVRQTPEKRLEWVAGISSGG SYTYYPDTLKGRFTVSRDNARNTLYLQMSSLRSEDTAKYFCASQGSNHYFDYWG



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QGTTLTVSS

SEQ ID NO: 49

1E4 LCVR

DTLMTQSPSSMSVSLGDTVSITCHASQGIHNNIGWLQQKPGKSFKALIYHGTNLED
GVPSRFSGSGSGADYSLIISSLESEDFADYYCVQYAQFPYTFGGGTKLEIK

SEQ ID NO: 50

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GFTFSRYAMS | SEQ ID NO: 51 |
| HCDR2 | GISSGGSYTYYPDTLKG | SEQ ID NO: 52 |
| HCDR3 | QGSNHYFDY | SEQ ID NO: 53 |
| LCDR1 | HASQGIHNNIG | SEQ ID NO: 54 |
| LCDR2 | HGTNLED | SEQ ID NO: 55 |
| LCDR3 | VQYAQFPYT | SEQ ID NO: 56 |

The heavy and light chain variable region sequences of murine mAb 2G6 are as follows:

2G6 HCVR

EVQLVESGGGLVKPGGSLKLSCAASGFTFSRYGMSWVRQTPEKRLEWVAGINGG
GSYTYYLDTVKGRFTISRDNSRNTLYLQMSSLRSEDTAMYYCVSQGSNYYFDYW
GQGTTLTVSS

SEQ ID NO: 57

2G6 LCVR

DIRMTQSPSSMSVSLGDTVSITCHASQGISSNIGWLQQKPGKSFKALIYHGTNLED
GVPSRFSGSGSGADYSLTISSLESEDFADYYCVQYAQFPYTFGGGTKLEIK

SEQ ID NO: 58

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GFTFSRYGMS | SEQ ID NO: 59 |
| HCDR2 | GINGGGSYTYYLDTVKG | SEQ ID NO: 60 |
| HCDR3 | QGSNYYFDY | SEQ ID NO: 61 |
| LCDR1 | HASQGISSNIG | SEQ ID NO: 62 |
| LCDR2 | HGTNLED | SEQ ID NO: 63 |
| LCDR3 | VQYAQFPYT | SEQ ID NO: 64 |

The heavy and light chain variable region sequences of murine mAb 1C9 are as



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follows:

1C9 HCVR

QVQLQQPGSVLVRPGASVKLSCKASGDTFTTYWMNWVKQRPGQGLEWIGGIYL
NSGSSEYNEKFKGKATLSVDTSSSTAYMDLSSLTSEDSAVYYCARDSRFSYWGQG
TLVTVSA

SEQ ID NO: 65

1C9 LCVR

DIVMTQSHKFLSTSVGDRVSITCKASQDVSTAVAWYQQKPGQSPELLISSASYRYT
GVPDRFTGSGSGTDFTFTISSVQAEDLAVYYCQQHYNTPLTFGAGTQLELK

SEQ ID NO: 66

It contains the following CDR sequences:

| Name | sequence | SEQ ID NO: |
|---|---|---|
| HCDR1 | GDTFTTY | SEQ ID NO: 67 |
| HCDR2 | YLNSGS | SEQ ID NO: 68 |
| HCDR3 | DSRFSY | SEQ ID NO: 69 |
| LCDR1 | KASQDVSTAVA | SEQ ID NO: 70 |
| LCDR2 | SASYRYT | SEQ ID NO: 71 |
| LCDR3 | QQHYNTPLT | SEQ ID NO: 72 |

The heavy and light chain variable regions of each mouse mAb were cloned into the human IgG1 heavy chain constant region and the kappa light chain constant region respectively, and then were purified, identified, and tested for activity as described in Example 4.

**Example 3: Detection of *in vitro* binding activity of the antibodies**

(1) Indirect ELISA binding assay *in vitro*:

HuB7-H4 His protein (Sino Biological Inc., cat#10738-H08H) was diluted to a concentration of 1 μg/ml with PBS, pH 7.4, added into a 96-well high-affinity microtiter plate at a volume of 100 μl/well, and incubated at 4°C overnight (16-20 hours). The plate was washed with PBST (PBS comprising 0.05% Tween-20, pH 7.4) four times, and then added with 150 μl/well of 3% bovine serum albumin (BSA) blocking solution diluted in PBST, and incubated at room temperature for 1 hour for blocking. After the blocking was finished, the blocking solution was discarded and the plate was washed 4 times with PBST buffer.

The antibodies to be tested were diluted with PBST comprising 3% BSA obtain a gradient of 10-fold dilution, starting from 1 μM with a total of 10 doses. The dilutions were added into the plate at 100 μl/well, and incubated at room temperature for 1 hour. After the incubation was finished, the plate was washed 4 times with PBST, added with



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100 μl/well of HRP-labeled goat anti-human secondary antibody (Abeam, cat#ab97225) diluted in PBST comprising 3% BSA, and incubated at room temperature for 1 hour. The plate was washed 4 times with PBST, added with 100 μl/well TMB chromogenic substrate (Cell Signaling Technology, cat#7004S), incubated at room temperature in darkness for 1 minute, and added with 100 μl/well of Stop Solution (Cell Signaling Technology, cat#7002S) to terminate the reaction. The absorbance was read at 450 nm using a microplate reader (BioTek, model Synergy H1), and the data was analyzed. The curve of concentration vs. signal value was plotted and the results were analyzed, as shown in the following table:

| Chimeric antibody | $EC_{50}$ for the binding to human B7-H4 His antigen (nM) |
|---|---|
| M1 | 0.063 |
| 2A10 | 0.071 |
| 2F4 | 0.056 |
| 2F7 | 0.17 |
| 2F8 | 0.067 |
| 2G6 | 0.081 |
| 1C9 | 0.068 |

(2) Competitive ELISA Assay:

HuB7-H4 His protein (Sino Biological Inc., cat#10738-H08H) was diluted to a concentration of 1 μg/ml with PBS, pH 7.4, added into a 96-well high-affinity microtiter plate at a volume of 100 μl/well, and incubated at 4°C overnight (16-20 hours). The plate was washed 4 times with PBST (PBS comprising 0.05% Tween-20, pH 7.4), added with 150 μl/well of 3% bovine serum albumin (BSA) blocking solution diluted in PBST, and incubated at room temperature for 1 hour. After the blocking was finished, the blocking solution was discarded and the plate was washed 4 times with PBST buffer.

0.1 nM reference chimeric antibody was prepared with PBST comprising 3% BSA, and was used as dilution solution to dilute the murine antibodies to be tested, obtain a gradient of 10-fold dilution, starting from 100 nM with a total of 10 doses. The diluted antibodies were added into the plate at 100 μl/well, and incubated for 1 hour at room temperature. After the incubation was finished, the plate was washed 4 times with PBST, and 100 μl/well of HRP-labeled goat anti-human secondary antibody (Abcam, cat#ab97225) diluted in PBST comprising 3% BSA was added, and incubated for 1 hour at room temperature. The plate was washed 4 times with PBST, and then 100 μl/well TMB chromogenic substrate (Cell Signaling Technology, cat#7004S) was added, and incubated at room temperature for 1 minute in darkness. 100 μl/well of Stop Solution (Cell Signaling Technology, cat#7002S) was added to terminate the reaction, and the absorbance was read at 450 nm using a microplate reader (BioTek, model Synergy H1). The data was analyzed. Competitive inhibition rate = ((reference antibody absorbance - competitive antibody



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absorbance) / Abv antibody absorbance) * 100.

(3) Capture ELISA binding assay *in vitro*:

The goat anti-mouse IgG secondary antibody (Jackson Immuno Research, cat #115-006-071) was diluted to a concentration of 2 μg/ml with PBS buffer, pH 7.4, added into a 96-well microtiter plate at a volume of 100 μl/well, and incubated in an incubator at 37°C for 2 hours. The plate was washed once with PBST, added with 5% skim milk (Bright Dairy, Skim Milk Powder) blocking solution diluted in PBST at 200 μl/well, and incubated at 37 °C for 2 hours or at 4 °C overnight (16-18 hours) for blocking. After the blocking was finished, the blocking solution was discarded and the plate was washed 4 times with PBST.

The mouse sera or purified recombinant antibodies to be tested were diluted to various concentrations with sample dilution comprising 5% NHS (PBST with 2.5% skim milk), incubated for 40 minutes at room temperature, added into a plate at 100 μl/well, and incubated in an incubator for 40 minutes at 37°C. After the incubation was finished, the plate was washed 4 times with PBST, added with 100 μl/well of biotinylated huB7-H4-his (Sino Biological #10738-H08H) protein solution diluted in sample dilution solution, and incubated at 37 °C for 40 minutes. After the incubation was finished, the plate was washed 4 times with PBST, added with 100 μl/well of HRP-labeled streptavidin (Jackson Immuno Research, cat #016-030-084) diluted in PBST, and incubated at 37°C for 40 minutes. After the plate was washed 4 times with PBST, 100 μl/well TMB chromogenic substrate (InnoReagents Biotechnology Co., Ltd.) was added, incubated at room temperature for 10-15 min in darkness, 50 μl/well 1M $H_2SO_4$ was added to stop the reaction. The absorbance was read at 450 nm using a microplate reader (Beijing Perlong New Technology Co., Ltd., model DNM-9602) and data was analyzed.

(4) Cell binding assay *in vitro*:

The cultured SK-BR3 cells or cells stably transfected with CHO-huB7-H4 were collected, and then plated onto a 96-well U-bottom plate at cell density of 1 x $10^5$ to 2 x $10^5$ cells per well. The supernatant was removed by centrifuging at 1200g for 5min, 100μl of serially diluted antibody solutions or mouse immune sera were added, and incubated at 4°C for 60min, and the supernatant was removed by centrifuging at 1200g for 5min. The cells were washed twice with PBS, added with fluorescent labeled secondary antibody (PE-GAM or PE-GAH) at 100 μl per well, incubated for 60 min at 4°C, and centrifuged at 1200 g for 5 min to remove the supernatant. The cells were washed twice with PBS, and then were re-suspended in PBS. The signals were detected using flow cytometer, and the concentration curve was plotted and results were analyzed.

**Example 4: Construction and expression of anti-B7-H4 recombinant chimeric antibodies**

Site-directed amino acid mutations were made in the FR region(s) (framework



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regions) of the heavy chain variable region (VH) and the light chain variable region (VL) for each murine antibody of the present invention. Different humanized antibody heavy and light chain were designed according to different combinations of amino acid mutations. Cells transfected with plasmids of various combinations of heavy and light chains would be used to produce humanized antibodies.

The heavy chain vector was designed as follows: signal peptide + mutated heavy chain variable region sequence + human IgG1 constant region sequence.

The light chain vector was designed as follows: signal peptide + mutated light chain variable region sequence + human Kappa constant region sequence.

The above sequences were inserted into pCEP4 vector respectively. Expression vectors were synthesized according to the above design, the resulting vector plasmids were extracted with maxi extraction kit, and were validated by sequencing. The validated plasmids were transfected into human 293F cells with PEI and cultured continuously. The 293F cells were cultured in serum-free medium (Shanghai opmbiosciences, OPM-293CD03) to logarithmic growth phase for cell transfection. 21.4 μg of the humanized antibody light chain plasmid and 23.6 μg of the humanized antibody heavy chain plasmid were dissolved in 10 ml of Opti-MEM® I Reduced Serum Medium(GIBCO, 31985-070), mixed well, then added with 200 μg of PEI, mixed well, incubated for 15 min at RT, and added into 50mL of cells. Cell culture conditions were as follows: 5% $CO_2$, 37 °C, 125 rpm/min. During the culture period, medium was replenished on day 1 and day 3, until the cell viability reached to less than 70%. The cell supernatant was collected and centrifuged for filtration. After centrifugation, the cell culture was applied to an affinity column for antibody purification. The purified chimeric antibodies were finally obtained after the column was washed with phosphate buffer, eluted with glycine hydrochloride buffer (0.1 M Gly-HCl, pH 2.7), neutralized with 1 M Tris hydrochloric acid pH 9.0, and dialyzed against phosphate buffer.

**Example 5: Binding affinity and kinetic assay *in vitro*:**

Biacore method is recognized as a method for the objective detection of the affinity and kinetics between proteins. The affinities and binding kinetics of the B7-H4 antibodies of the invention to be tested were analyzed by Biacore T200 (GE).

The anti-B7-H4 antibody of the present invention to be tested was covalently linked to CM5 (GE) chip by NHS standard amino coupling method using a kit provided by Biacore. Then,

a). 50 nM human huB7-H4-his protein (Sino Biological #10738-H08H) diluted by the same buffer was loaded at a flow rate of 10 μL/min, and the chip was regenerated with the regeneration reagent provided in the kit. Antigen-antibody binding kinetics were recorded for 3 minutes and dissociation kinetics were recorded for 10 minutes. The resulting data were analyzed by GE's BIAevaluation Software using 1:1 (Langmuir)



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binding model. The kd (koff) data of each murine antibody estimated by this method were shown in the following table.

| Murine antibody | antigen | dissociation rate $k_d$ (1/s) |
|---|---|---|
| 2F7 | huB7-H4-his | 2.53E-03 |
| 2F8 | | 6.87E-05 |
| M1 | | 3.98E-05 |
| 2A10 | | 3.31E-04 |
| 2G6 | | 8.91E-05 |
| 2F4 | | 1.66E-03 |
| 2E4 | | 5.97E-05 |
| 1E4 | | 2.93E-04 |

b). A series of concentrations of human huB7-H4-his protein diluted by the same buffer were respectively loaded at the flow rate of 10 μL/min and the chip was regenerated with the regeneration reagent provided in the kit. Antigen-antibody binding kinetics were recorded for 3 minutes and dissociation kinetics were recorded for 10 minutes. The resulting data were analyzed by GE's BIAevaluation Software using 1:1 (Langmuir) binding model. The ka(kon), kd (koff) and $K_D$ value for each chimeric antibody estimated by this method were shown in the following table.

| chimeric antibody | antigen | Association rate $k_a$(1/M*s) | dissociation rate $k_d$ (1/s) | affinity $K_D$ |
|---|---|---|---|---|
| ch-2F7 | huB7-H4-his | 4.417E+04 | 3.038E-03 | 68.8nM |
| ch-2F8 | | 1.126E+06 | 1.136E-04 | 119pM |
| ch-M1 | | 6.743E+05 | 9.411E-05 | 140pM |
| ch-2F4 | | 1.040E+06 | 2.593E-03 | 2.5nM |

**Example 6: Humanization of mouse antibodies**

Humanization of murine anti-human B7-H4 monoclonal antibodies was performed as disclosed in many literatures in the art. Briefly, the parental (murine antibody) constant domain was replaced with human constant domain, and the human antibody sequences were selected on the basis of the homology between the murine antibody and the human antibody. In the present invention, the murine candidate molecules 2F7, 2F8, 2G6 and 1C9 were subjected to humanization.

Based on the typical structure of the resulting murine antibody VH/VL CDR, the



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heavy and light chain variable region sequences were compared with the human antibody germline database to obtain human germline template with high homology.

The CDR regions of the murine antibodies 2F7, 2F8, 2G6 and 1C9 were grafted onto the selected corresponding humanized template. For 2F8, the HCDR1 region (GYTFTNSWMN, SEQ ID NO: 19) and HCDR2 region (GIYPNSGNIEYNEKFKG, SEQ ID NO: 20) were replaced with GYTFTSSWMN(SEQ ID NO: 73) and GIYPNRGNIEYNEKFKG(SEQ ID NO: 74), respectively, to remove potential unstable de-acetylation sites. The humanized variable regions were replaced and recombined with IgG constant region (preferably IgG1 for the heavy chain and κ for the light chain). Then, based on the three-dimensional structure of the murine antibody, back mutations were made on the embedded residues, residues which directly interacted with the CDRs and the residues which have an important impact on the conformation of VL and VH. The chemically instable amino acid residues in CDR regions were optimized. The antibodies resulting from the combination of humanized light and heavy chain variable region sequences were obtained and detected.

The humanized hu2F7, hu2F8, hu2G6 and hu1C9 antibody molecules were finally selected by expression test and comparison of the number of back mutations, and the respective heavy and light chain variable region sequences were shown in SEQ ID NOs: 75-82, and the respective heavy and light chain sequences were shown in SEQ ID NOs: 83-90.

hu2F7 HCVR

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWVRQAPGKGLEWVAYVSSG GGSTYYSDSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCTRESYSQGNYFDY WGQGTTVTVSS

SEQ ID NO: 75

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWVRQAPGKGLEWVAYVSSG GGSTYYSDSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCARESYSQGNYFDY WGQGTTVTVSS

SEQ ID NO: 91

hu2F7 LCVR

EIVMTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQSPRLLIKFASQSISGIP ARFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFSLTFGQGTKLEIK

SEQ ID NO: 76

EIVLTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQAPRLLIYFASQSISGIP ARFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFSLTFGQGTKLEIK

SEQ ID NO: 92



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EIVLTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQAPRLLIKFASQSISGIP
ARFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFSLTFGQGTKLEIK

SEQ ID NO: 93

EIVMTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQAPRLLIKFASQSISGI
PARFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFSLTFGQGTKLEIK

SEQ ID NO: 94

hu2F8 HCVR

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSSWMNWVRQAPGQRLEWMGGIYP
NRGNIEYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQG
TLVTVSS

SEQ ID NO: 77

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSSWMNWVRQAPGQRLEWMGGIYP
NRGNIEYNEKFKGRVTITVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQG
TLVTVSS

SEQ ID NO: 95

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSSWMNWVRQAPGQGLEWMGGIYP
NRGNIEYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQG
TLVTVSS

SEQ ID NO: 96

hu2F8 LCVR

DIQMTQSPSSLSASVGDRVTITCKASQDVRTAVAWYQQKPGKAPKLLISSTSYRYT
GVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYSTPLTFGGGTKVEIK

SEQ ID NO: 78

DIVMTQSPSSLSASVGDRVTITCKASQDVRTAVAWYQQKPGKAPKLLISSTSYRYT
GVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYSTPLTFGGGTKVEIK

SEQ ID NO: 97

DIVMTQSPSSLSASVGDRVTITCKASQDVRTAVAWYQQKPGKSPKLLISSTSYRYTG
VPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYSTPLTFGGGTKVEIK

SEQ ID NO: 98

hu2G6 HCVR

EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQAPGKGLEWVSGINGG
GSYTYYLDTVKGRFTISRDNARNTLYLQMSSLRAEDTAVYYCVSQGSNYYFDYW
GQGTLVTVSS

SEQ ID NO: 79



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hu2G6 LCVR

DIRMTQSPSSLSASVGDRVTITCHASQGISSNIGWLQQKPGKAPKALIYHGTNLED
GVPSRFSGSGSGADYTLTISSLQPEDFATYYCVQYAQFPYTFGGGTKVEIK

SEQ ID NO: 80

hu1C9 HCVR

EVQLVQSGAEVKKPGASVKVSCKASGDTFTTYWMNWVRQAPGQRLEWMGGIY
LNRGSSEYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQ
GTLVTVSS

SEQ ID NO: 81

hu1C9 LCVR

DIQMTQSPSSLSASVGDRVTITCKASQDVSTAVAWYQQKPGKAPKLLISSASYRYT
GVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYNTPLTFGGGTKVEIK

SEQ ID NO: 82

hu2F7 HC

EVQLVESGGGLVQPGGSLRLSCAASGFTFSNYYMSWVRQAPGKGLEWVAYVSSG
GGSTYYSDSVKGRFTISRDNAKNTLYLQMSSLRAEDTAVYYCTRESYSQGNYFDY
WGQGTTVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSG
ALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEP
KSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV
KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
KALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWE
SNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHY
TQKSLSLSPGK

SEQ ID NO: 83

hu2F7 LC

EIVMTQSPATLSLSPGERATLSCRASQSISDYLHWYQQKPGQSPRLLIKFASQSISGIP
ARFSGSGSGTDFTLTISSLEPEDFAVYYCQNGHSFSLTFGQGTKLEIKRTVAAPSVFI
FPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDST
YSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 84

hu2F8 HC

EVQLVQSGAEVKKPGASVKVSCKASGYTFTSSWMNWVRQAPGQRLEWMGGIYP
NRGNIEYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQG
TLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSG
VHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCDK



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THTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWY
VDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPI
EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE
NNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL
SPGK

SEQ ID NO: 85

hu2F8 LC

DIQMTQSPSSLSASVGDRVTITCKASQDVRTAVAWYQQKPGKAPKLLISSTSYRYT
GVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYSTPLTFGGGTKVEIKRTVAAPS
VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 86

hu2G6 HC

EVQLLESGGGLVQPGGSLRLSCAASGFTFSRYGMSWVRQAPGKGLEWVSGINGG
GSYTYYLDTVKGRFTISRDNARNTLYLQMSSLRAEDTAVYYCVSQGSNYYFDYW
GQGTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGA
LTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPK
SCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKF
NWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKA
LPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES
NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYT
QKSLSLSPGK

SEQ ID NO: 87

hu2G6 LC

DIRMTQSPSSLSASVGDRVTITCHASQGISSNIGWLQQKPGKAPKALIYHGTNLED
GVPSRFSGSGSGADYTLTISSLQPEDFATYYCVQYAQFPYTFGGGTKVEIKRTVAAP
SVFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDS
KDSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 88

hu1C9 HC

EVQLVQSGAEVKKPGASVKVSCKASGDTFTTYWMNWVRQAPGQRLEWMGGIY
LNRGSSEYNEKFKGRVTLTVDTSASTAYMELSSLRSEDTAVYYCARDSRFSYWGQ
GTLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTS
GVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKVEPKSCD
KTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNW



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YVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA
PIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQP
ENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLS
LSPGK

SEQ ID NO: 89

hu1C9 LC

DIQMTQSPSSLSASVGDRVTITCKASQDVSTAVAWYQQKPGKAPKLLISSASYRYT
GVPSRFSGSGSGTDFTFTISSLQPEDIATYYCQQHYNTPLTFGGGTKVEIKRTVAAPS
VFIFPPSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSK
DSTYSLSSTLTLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

SEQ ID NO: 90

cDNA fragments were synthesized based on the amino acid sequences of the above humanized antibody light and heavy chains, and inserted into pcDNA3.1 expression vectors (Life Technologies Cat. No. V790-20). The expression vectors and the transfection reagent PEI (Polysciences, Inc. Cat. No. 23966) were transfected into HEK293 cells (Life Technologies Cat. No. 11625019) at a ratio of 1:2 and incubated in a $CO_2$ incubator for 4-5 day. The expressed antibodies were recovered by centrifugation, and antibody purification was carried out in accordance with the method of Example 4 to obtain humanized antibody proteins hu2F7 and hu2F8 of the present invention.

**Example 7: Determination of activity of humanized antibodies**

The following *in vitro* assays were performed on humanized antibodies hu2F7 and hu2F8:

1. Cell binding assay *in vitro*:

The cultured MX-1 cells were collected, cell density was adjusted with PBS pH 7.4, then plated onto a 96-well V-shaped bottom plate with 1 x $10^5$ cells per well, and centrifuged at 2000 rpm for 5 minutes to remove supernatant. 100 μl of the serially diluted chimeric antibody solution was added (diluted with 0.5% BSA in PBS to obtain a gradient of 3-fold dilutions, starting from 1 μM, with a total of 10 doses) into each well, mixed, and incubated for 1 hour at 4 °C with shaking; the culture was centrifuged at 2000 rpm for 5 minutes to remove the supernatant, and the cells were washed twice with PBS, and each well was added with 100 μl of FITC-labeled goat anti-human secondary antibody (Abeam, cat#ab97224) diluted with 0.5% BSA in PBS, mixed well and incubated for 30 minutes at 4°C in a shaker. Centrifugation was performed at 2000 rpm for 5 minutes and the supernatant was removed. Cells were washed twice with PBS and resuspended in PBS. The signals were detected using flow cytometer (BECKMANCOULTER, model DxFLEX), and the concentration curve was plotted and the results were analyzed. As indicated in the table and figure, humanized antibodies 2F7, 2F8, 2G6 and 1C9 show



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positive bindings to MX-1 cells on which B7-H4 was highly expressed.

| Name of antibody | FACS Binding $EC_{50}$(nM) for MX-1 cell |
|---|---|
| hu2F7 | 7.32 |

| Name of antibody | FACS Binding $EC_{50}$(nM) for MX-1 cell |
|---|---|
| hu2F8 | 7.26 |

| Name of antibody | FACS Binding $EC_{50}$(nM) for MX-1 cell |
|---|---|
| hu2G6 | 14.3 |

| Name of antibody | FACS Binding $EC_{50}$(nM) for MX-1 cell |
|---|---|
| hu1C9 | 7.25 |

2. Affinity kinetic assay (method procedures were the same as those described in Example 5). The results were shown in the table below. The humanized antibodies hu2F7, hu2F8, hu2G6 and hu1C9 all show strong affinity for the human B7-H4 antigen protein.

| antibody | antigen | Association rate $k_a$(1/M*s) | Dissociation rate $k_d$ (1/s) | affinity $K_D$ |
|---|---|---|---|---|
| hu2F7 | hu-B7-H4-his | 3.29e+05 | 2.49e-04 | 7.57e-10 |
| hu2F8 | | 4.47e+05 | 1.12e-04 | 2.50e-10 |
| hu2G6 | | 7.41e+05 | 1.00e-05 | 1.35e-11 |
| hu1C9 | | 3.35e+05 | 1.00e-05 | 2.98e-11 |

**Example 8: Determination of binding epitopes recognized by the humanized antibodies**

The method procedures were the same as described in Example 3 (1): Indirect ELISA binding assay *in vitro*. The B7-H4 of SEQ ID NO: 92 was degraded into antigen fragments of 20 amino acids in length, and the antigen fragment P12 (amino acid sequence: TVASAGNIGEDGILSCTFEP) was found to be specifically bound by the humanized anti-B7-H4 antibody hu2F7, in indirect ELISA binding assay *in vitro*. The results were shown in Figure 2. To further confirm the epitope to which the antibody binds, alanine scan was performed on the antigen fragment P12, that is, each single amino acid located in



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P12 was mutated to alanine respectively. It was found in indirect ELISA binding assay *in vitro* that mutations in amino acid sequence ILSCTFE portion significantly attenuated binding of the antibody to the antigen fragment, showing that the antibody binding epitope was located in the amino acid sequence portion of ILSCTFE (SEQ ID NO: 109) comprised in the amino acid sequence TVASAGNIGEDGILSCTFEP. The results were shown in the following table:

| Antigen fragment sequence | SEQ ID NO: | Affinity EC50 (nM) |
|---|---|---|
| TVASAGNIGEDGILSCTFEP | 101 | 117.9 |
| TVASAGNIGEDGALSCTFEP | 102 | 770.6 |
| TVASAGNIGEDGIASCTFEP | 103 | 1476 |
| TVASAGNIGEDGILSATFEP | 104 | 2006 |
| TVASAGNIGEDGILSCAFEP | 105 | 462.8 |
| TVASAGNIGEDGILSCTAEP | 106 | 986.5 |
| TVASAGNIGEDGILSCTFAP | 107 | 662.5 |

It was found in indirect ELISA binding assay *in vitro* that the antigen fragment P12 (amino acid sequence: TVASAGNIGEDGILSCTFEP) was specifically bound by the humanized anti-B7-H4 antibody, hu1C9. The results were shown in the table below. To further confirm the epitope to which the antibody binds, alanine scan was performed on the antigen fragment P12, that is, each single amino acid located in P12 was mutated to alanine respectively. It was found in indirect ELISA binding assay *in vitro* that mutations in amino acid sequence LSCTF portion significantly attenuated binding of the antibody to the antigen fragment, showing that the antibody binding epitope was located in the amino acid sequence portion of LSCTF (SEQ ID NO: 110) comprised in the amino acid sequence TVASAGNIGEDGILSCTFEP. The results were shown in the following table:

| Antigen fragment sequence | SEQ ID NO: | Affinity EC50 (nM) |
|---|---|---|
| TVASAGNIGEDGILSCTFEP | 101 | 271.7 |
| TVASAGNIGEDGIASCTFEP | 103 | 466.9 |
| TVASAGNIGEDGILSATFEP | 104 | 292.3 |
| TVASAGNIGEDGILSCTAEP | 106 | 487.9 |

It was found in indirect ELISA binding assay *in vitro* that the antigen fragment P12 (amino acid sequence: TVASAGNIGEDGILSCTFEP) was specifically bound by the humanized anti-B7-H4 antibody, hu2G6. The results were shown in the table below. To further confirm the epitope to which the antibody binds, alanine scan was performed on the antigen fragment P12, that is, each single amino acid located in P12 was mutated to alanine respectively. It was found in indirect ELISA binding assay *in vitro* that mutations



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in amino acid sequence ILSCTFEP portion significantly attenuated binding of the antibody to the antigen fragment, showing that the antibody binding epitope was located in the amino acid sequence portion of ILSCTFEP (SEQ ID NO: 111) comprised in the amino acid sequence TVASAGNIGEDGILSCTFEP. The results were shown in the following table:

| Antigen fragment sequence | SEQ ID NO: | Affinity EC50 (nM) |
|---|---|---|
| TVASAGNIGEDGILSCTFEP | 101 | 792.6 |
| TVASAGNIGEDGALSCTFEP | 102 | 2626 |
| TVASAGNIGEDGIASCTFEP | 103 | 3158 |
| TVASAGNIGEDGILSATFEP | 104 | 3480 |
| TVASAGNIGEDGILSCAFEP | 105 | 2289 |
| TVASAGNIGEDGILSCTAEP | 106 | 2600 |
| TVASAGNIGEDGILSCTFEA | 108 | 2372 |

**Example 9: Anti-tumor effect of humanized antibodies**

MC38 tumor cells were implanted into mice into which human B7-H4 gene was introduced by gene editing technology. When the tumor reached an average of 100 $mm^3$, the mice were injected with control or humanized B7-H4 antibody hu1C9 (10mg/kg or 30mg/kg) every 3 days. By observing the size of the tumor, it was found that hu1C9 has a significant effect on inhibiting tumor growth and has an anti-tumor effect. The specific results were shown in Figure 3 and the following table:

| | **Volume of the tumor (mean, $mm^3$)** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **Day 0** | **Day 3** | **Day 6** | **Day 9** | **Day 12** | **Day 15** | **Day 18** |
| Blank control group | 136.2979 | 290.2921 | 526.7519 | 820.9497 | 1689.954 | 3625.686 | 5913.385 |
| 1C9 (10mg/kg) | 146.5273 | 231.8861 | 404.833 | 794.0959 | 1519.589 | 1717.092 | 2326.687 |
| 1C9 (30mg/kg) | 139.3148 | 256.9988 | 437.9834 | 702.0555 | 1190.775 | 2008.451 | 2744.415 |

**Example 10: Effect of humanized antibodies on enhancing immunity**

The anti-tumor effect of anti-B7-H4 may be mediated by enhancing immune effects against tumors. To validate this mechanism, CD4-positive T cells were extracted from human peripheral blood. Various concentrations of anti-B7-H4 antibodies, including the humanized antibody hu2G6 and hu1C9, were added into the cultured T cells. The specific results were shown in Fig. 4 and the table below. The humanized antibody hu2G6 and the humanized antibody hu1C9 have an effect on enhancing proliferation of T cells, and can achieve an antitumor effect by enhancing immune effects against tumors.



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| | Proliferation of T cells (percentage, mean) | | |
|---|---|---|---|
| dosage | 1C9 | 2G6 | Blank control group |
| 0.1 (μg/ml) | 62.49425 | 53.6423 | -12.7833 |
| 0.3 (μg/ml) | 76.83938 | 76.88089 | -4.12145 |
| 3 (μg/ml) | 86.96902 | 85.86148 | -4.39787 |



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**What is claimed is**:

1. An anti-B7-H4 antibody or antigen-binding fragment thereof, comprising:
antibody light chain variable region comprising at least one LCDR selected from the group consisting of:
SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8;
SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16;
SEQ ID NO:22, SEQ ID NO:23, SEQ ID NO:24;
SEQ ID NO:30, SEQ ID NO:31, SEQ ID NO:32;
SEQ ID NO:38, SEQ ID NO:39, SEQ ID NO:40;
SEQ ID NO:46, SEQ ID NO:47, SEQ ID NO:48;
SEQ ID NO:54, SEQ ID NO:55, SEQ ID NO:56;
SEQ ID NO: 62, SEQ ID NO: 63, SEQ ID NO: 64;
SEQ ID NO: 70, SEQ ID NO: 71 SEQ ID NO: 72; and
antibody heavy chain variable region comprising at least one HCDR selected from the group consisting of:
SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5;
SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13;
SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21;
SEQ ID NO:27, SEQ ID NO:28, SEQ ID NO:29;
SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37;
SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45;
SEQ ID NO: 51, SEQ ID NO: 52, SEQ ID NO: 53;
SEQ ID NO:59, SEQ ID NO:60, SEQ ID NO:61;
SEQ ID NO: 67, SEQ ID NO: 68, SEQ ID NO: 69;
SEQ ID NO:73 and SEQ ID NO:74.

2. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody heavy chain variable region comprises:
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 3, SEQ ID NO: 4 and SEQ ID NO: 5, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 19, SEQ ID NO: 20 and SEQ ID NO: 21, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 43, SEQ ID NO: 44 and



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SEQ ID NO: 45, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 51, SEQ ID NO: 52 and SEQ ID NO: 53, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 59, SEQ ID NO: 60 and SEQ ID NO: 61, respectively;
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 67, SEQ ID NO: 68 and SEQ ID NO: 69, respectively; or
HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:73, SEQ ID NO:74 and SEQ ID NO:21, respectively.

3. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises:
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 46, SEQ ID NO: 47 and SEQ ID NO: 48, respectively;
LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 54, SEQ ID NO: 55 and SEQ ID NO: 56, respectively;
LCDR1, LCDR2, and LCDR3 as shown in SEQ ID NO: 62, SEQ ID NO: 63, and SEQ ID NO: 64, respectively; or
LCDR1, LCDR2, and LCDR3 as shown in SEQ ID NO: 70, SEQ ID NO: 71, and SEQ ID NO: 72, respectively.

4. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 6, SEQ ID NO: 7 and SEQ ID NO: 8, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:3, SEQ ID NO:4 and SEQ ID NO:5, respectively.

5. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 14, SEQ ID NO: 15 and SEQ ID NO: 16,



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respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 11, SEQ ID NO: 12 and SEQ ID NO: 13, respectively.

6. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 19, SEQ ID NO: 20 and SEQ ID NO:21, respectively.

7. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 30, SEQ ID NO: 31 and SEQ ID NO: 32, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:29, respectively.

8. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 38, SEQ ID NO: 39 and SEQ ID NO: 40, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 35, SEQ ID NO: 36 and SEQ ID NO: 37, respectively.

9. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 46, SEQ ID NO: 47 and SEQ ID NO: 48, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 43, SEQ ID NO: 44 and SEQ ID NO: 45, respectively.

10. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 54, SEQ ID NO: 55 and SEQ ID NO: 56, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO: 51, SEQ ID NO: 52 and SEQ ID NO: 53, respectively.

11. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1,



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wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 62, SEQ ID NO: 63 and SEQ ID NO: 64, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:59, SEQ ID NO:60 and SEQ ID NO:61, respectively.

12. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 70, SEQ ID NO: 71 and SEQ ID NO: 72, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 as shown in SEQ ID NO:67, SEQ ID NO:68 and SEQ ID NO:69, respectively.

13. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 1, wherein said antibody light chain variable region comprises LCDR1, LCDR2 and LCDR3 as shown in SEQ ID NO: 22, SEQ ID NO: 23 and SEQ ID NO: 24, respectively; and the antibody heavy chain variable region comprises HCDR1, HCDR2 and HCDR3 shown in SEQ ID NO:73, SEQ ID NO:74 and SEQ ID NO:21, respectively.

14. The anti-B7-H4 antibody or antigen-binding fragment thereof according to any one of claims 1 to 13, wherein the antibody or antigen-binding fragment thereof is selected from the group consisting of murine antibody or fragment thereof, chimeric antibody or fragment thereof, human antibody or fragment thereof and humanized antibody or fragment thereof.

15. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 14, wherein the humanized antibody heavy chain variable region further comprises heavy chain FR region(s) of human IgG1, IgG2, IgG3 or IgG4 or a variant thereof, preferably comprises heavy chain FR region(s) of human IgG1, IgG2 or IgG4; more preferably comprises heavy chain FR region(s) of IgG1 which has been subjected to amino acid mutation to enhance ADCC toxicity.

16. The anti-B7-H4 antibody or antigen-binding fragment thereof of claim 14, wherein the humanized antibody light chain variable region is a light chain variable region comprising sequence selected from the group consisting of: SEQ ID NO: 76, SEQ ID NO :78, SEQ ID NO: 80 or SEQ ID NO: 82, preferably SEQ ID NO: 76 or SEQ ID NO: 80.

17. The anti-B7-H4 antibody or antigen-binding fragment thereof of claim 14,



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wherein the humanized antibody heavy chain variable region is a heavy chain variable region comprising sequence selected from the group consisting of: SEQ ID NO: 75, SEQ ID NO :77, SEQ ID NO: 79 or SEQ ID NO: 81, preferably SEQ ID NO: 75 or SEQ ID NO: 79.

18. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 14, wherein the humanized antibody light chain is a light chain comprising sequence selected from the group consisting of: SEQ ID NO: 84, SEQ ID NO: 86, SEQ ID NO: 88 or SEQ ID NO: 90, preferably SEQ ID NO: 84 or SEQ ID NO: 88.

19. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 14, wherein the humanized antibody heavy chain is a heavy chain comprising sequence selected from the group consisting of: SEQ ID NO: 83, SEQ ID NO: 85, SEQ ID NO: 87 or SEQ ID NO: 89, preferably SEQ ID NO: 83 or SEQ ID NO: 87.

20. The anti-B7-H4 antibody or antigen-binding fragment thereof according to claim 14, wherein the humanized antibody comprises:
(1) light chain variable region of SEQ ID NO: 76 and heavy chain variable region of SEQ ID NO: 75;
(2) light chain variable region of SEQ ID NO: 78 and heavy chain variable region of SEQ ID NO: 77;
(3) light chain variable region of SEQ ID NO: 80 and heavy chain variable region of SEQ ID NO: 79; or
(4) light chain variable region of SEQ ID NO: 82 and heavy chain variable region of SEQ ID NO: 81.

21. The anti-B7-H4 antibody or antigen-binding fragment thereof of claim 14, wherein the humanized antibody comprises:
(1) light chain of SEQ ID NO: 84 and heavy chain of SEQ ID NO: 83;
(2) light chain of SEQ ID NO: 86 and heavy chain of SEQ ID NO: 85;
(3) light chain of SEQ ID NO: 88 and heavy chain of SEQ ID NO: 87; or
(4) light chain of SEQ ID NO: 90 and heavy chain of SEQ ID NO:89.

22. An anti-B7-H4 antibody or antigen-binding fragment thereof having at least one of the following characteristics: (1) binding to an epitope comprising amino acids 41-60 in SEQ ID NO: 92 of B7-H4; and (2) binding to an epitope comprising amino acids 53-59 in SEQ ID NO: 92 of B7-H4.

23. An anti-B7-H4 antibody or antigen-binding fragment thereof having at least one of the following characteristics: (1) binding to an epitope comprising amino acid 53 in



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SEQ ID NO: 92 of B7-H4; (2) binding to an epitope comprising amino acid 54 in SEQ ID NO: 92 of B7-H4; (3) binding to an epitope comprising amino acid 56 in SEQ ID NO: 92 of B7-H4; (4) binding to an epitope comprising amino acid 57 in SEQ ID NO: 92 of B7-H4; (5) binding to an epitope comprising amino acid 58 in SEQ ID NO: 92 of B7-H4; and (6) binding to an epitope comprising amino acid 59 in SEQ ID NO: 92 of B7-H4.

24. A DNA sequence, encoding the antibody or antigen-binding fragment of any one of claims 1-23.

25. An expression vector, comprising the DNA sequence of claim 24.

26. A host cell, being introduced with or comprising the expression vector of claim 25.

27. The host cell according to claim 26, wherein said host cell is bacterium, preferably *Escherichia coli.*

28. The host cell according to claim 26, wherein the host cell is yeast, preferably *Pichia pastoris.*

29. The host cell according to claim 26, wherein the host cell is mammalian cell, preferably Chinese hamster ovary (CHO) cell or human embryonic kidney (HEK) 293 cell.

30. A method of producing an antibody comprising: culturing the host cell of claims 26-29, isolating the antibody from the culture, and purifying the antibody.

31. A pharmaceutical composition comprising the anti-B7-H4 antibody or antigen-binding fragment thereof of any one of claims 1 to 23, and a pharmaceutically acceptable excipient, diluent or carrier.

32. A detection reagent, comprising the anti-B7-H4 antibody or antigen-binding fragment thereof of any one of claims 1 to 23.

33. A diagnostic agent, comprising the anti-B7-H4 antibody or antigen-binding fragment thereof of any of claims 1-23.

34. Use of the anti-B7-H4 antibody or antigen-binding fragment thereof according to any one of claims 1 to 23, or the pharmaceutical composition according to claim 31 in



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the manufacture of a medicament for the treatment or prevention of B7-H4 mediated disease or condition; wherein the disease is preferably a cancer; preferably, the disease is B7-H4 expressing cancer; the cancer is most preferably selected from the group consisting of breast cancer, ovarian cancer, prostate cancer, pancreatic cancer, kidney cancer, lung cancer, liver cancer, stomach cancer, colon cancer, bladder cancer, esophageal cancer, cervical cancer, gallbladder cancer, glioblastoma and melanoma.



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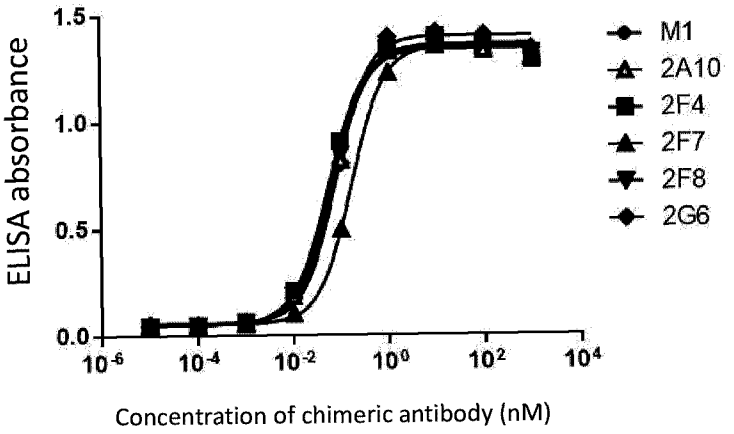


Fig 1

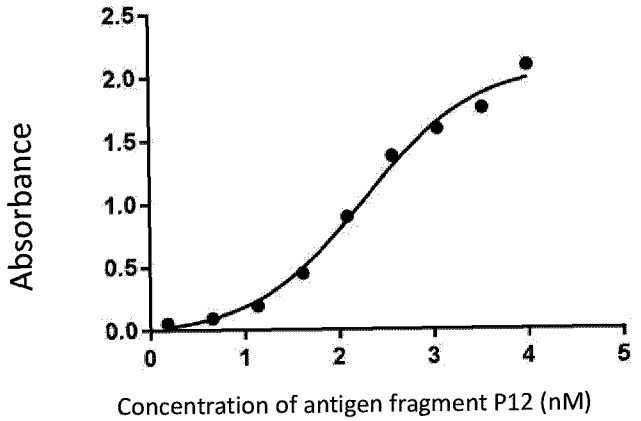


Fig 2

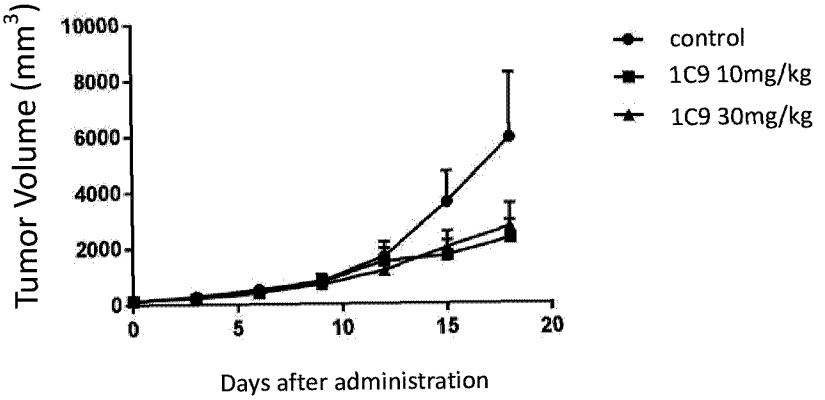


Fig 3



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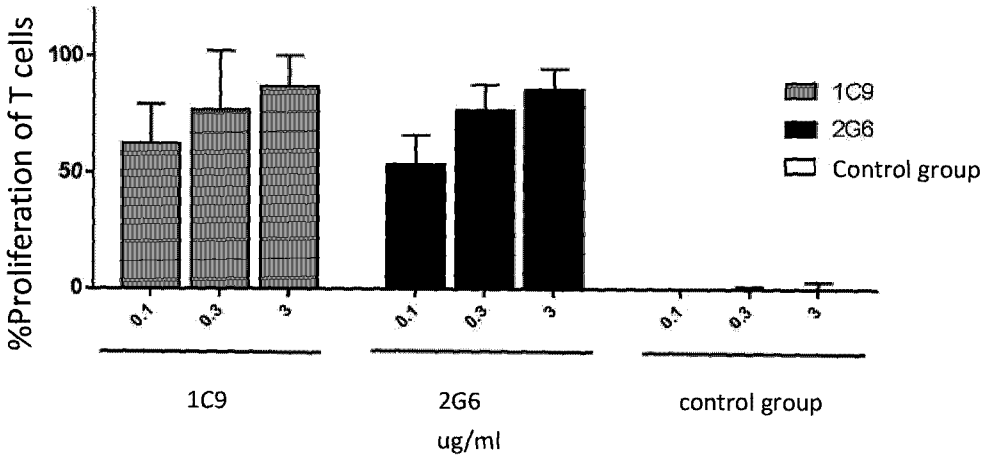


Fig 4



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