(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
19 April 2018 (19.04.2018)

(10) International Publication Number
WO 2018/068756 A1

(51) International Patent Classification:
C12N 15/85 (2006.01) *C12N 15/12* (2006.01)
C12N 15/90 (2006.01) *A01K 67/027* (2006.01)
C12N 15/113 (2010.01) *A61K 39/395* (2006.01)
C07K 14/705 (2006.01) *A61P 35/00* (2006.01)

(21) International Application Number: PCT/CN2017/106024

(22) International Filing Date: 13 October 2017 (13.10.2017)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
201610895750.9 14 October 2016 (14.10.2016) CN
201710948551.4 12 October 2017 (12.10.2017) CN

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: GENETICALLY MODIFIED NON-HUMAN ANIMAL WITH HUMAN OR CHIMERIC BTLA

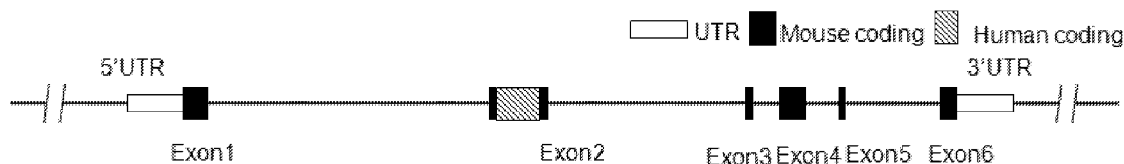

FIG. 3B

(57) Abstract: Genetically modified non-human animals that express a human or chimeric (e.g., humanized) B-and T-Lymphocyte-Associated Protein (BTLA or CD272), and methods of use thereof are provided.

GENETICALLY MODIFIED NON-HUMAN ANIMAL WITH HUMAN OR CHIMERIC BTLA

CLAIM OF PRIORITY

This application claims the benefit of Chinese Patent Application App. No. 201610895750.9, filed on October14,2016, and Chinese Patent Application App. No. 201710948551.4, filed on October12,2017. The entire contents of the foregoing are incorporated herein by reference.

TECHNICAL FIELD

This disclosure relates to genetically modified animal expressing human or chimeric (e.g., humanized) B- and T-Lymphocyte-Associated Protein(BTLA or CD272), and methods of use thereof.

BACKGROUND

The immune system has developed multiple mechanisms to prevent deleterious activation of T cells. One such mechanism is the intricate balance between positive and negative co-stimulatory signals delivered to T cells. Targeting the inhibitory pathways for the immune systemis considered to be a potential approach for the treatment of various diseases, e.g., cancers, and autoimmune diseases.

The traditional drug research and development for these inhibitory receptors typically use *in vitro* screening approaches. However, these screening approaches cannot provide the body environment (such as tumor microenvironment, stromal cells, extracellular matrix components and immune cell interaction, etc.), resulting in a higher rate of failure in drug development. In addition, in view of the differences between humans and animals, the test results obtained from the use of conventional experimental animals for *in vivo* pharmacological test may not be able to reflect the real disease state and the identification and interaction at the targeting sites, resulting in that the results in many clinical trials are significantly different from the animal experimental results. Therefore, the development of humanized animal models that are suitable for human

antibody screening and evaluation will significantly improve the efficiency of new drug development and reduce the costs for drug research and development.

SUMMARY

This disclosure is related to BTLA humanized animal model. The animal model can express human BTLA or chimeric BTLA (e.g., humanized BTLA) protein in its body. It can be used in the studies on the function of BTLA gene, and can be used in the screening and evaluation of anti-human BTLA antibodies. In addition, the animal models prepared by the methods described herein can be used in drug screening, pharmacodynamics studies, treatments for immune-related diseases (e.g., autoimmune disease), and cancer therapy for human BTLA target sites; in addition, they can be used to facilitate the development and design of new drugs, and save time and cost. In summary, this disclosure provides a powerful tool for studying the function of BTLA protein and screening for cancer drugs.

Furthermore, the disclosure also provides BTLA gene knockout mice. Moreover, the mice described in the present disclosure can be mated with the mice containing other human or chimeric genes (e.g., chimeric PD-1 or other immunomodulatory factors), so as to obtain a mouse expressing two or more human or chimeric proteins. The mice can also, e.g., be used for screening antibodies in the case of a combined use of drugs, as well as evaluating the efficacy of the combination therapy.

In one aspect, the disclosure relates to genetically-modified, non-human animals whose genome comprises at least one chromosome comprising a sequence encoding a human or chimeric B and T Lymphocyte Associated (BTLA or CD272). In some embodiments, the sequence encoding the human or chimeric BTLA is operably linked to an endogenous regulatory element at the endogenous BTLA gene locus in the at least one chromosome. In some embodiments, the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that is at least 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to human BTLA (NP_861445.3 (SEQ ID NO: 27)). In some embodiments, the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that is at least 50%, 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to

SEQ ID NO: 31.In some embodiments, the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that corresponds to amino acids 34-132 of SEQ ID NO: 27.

In some embodiments, the animal is a mammal, e.g., a monkey, a rodent or a mouse. In some embodiments, the animal is a C57BL/6 mouse.In some embodiments, the animal does not express endogenous BTLA. In some embodiments, the animal has one or more cells expressing human or chimeric BTLA. In some embodiments, the animal has one or more cells expressing human or chimeric BTLA, and the expressed human or chimeric BTLA can bind to or interact with humanherpes virus entry mediator (HVEM) or V-Set Domain Containing T-Cell Activation Inhibitor 1 (VTCN1 or B7-H4). In some embodiments, the animal has one or more cells expressing human or chimeric BTLA, and the expressed human or chimeric BTLA can bind to or interact with endogenous HVEM or B7-H4.

In one aspect, the disclosure relates to genetically-modified, non-human animals, wherein the genome of the animals comprises a replacement, at an endogenous BTLA gene locus, of a sequence encoding a region of endogenous BTLA with a sequence encoding a corresponding region of human BTLA.In some embodiments, the sequence encoding the corresponding region of human BTLA is operably linked to an endogenous regulatory element at the endogenous BTLA locus, and one or more cells of the animal expresses a chimeric BTLA. In some embodiments, the animal does not express endogenous BTLA. In some embodiments, the region of endogenous BTLA is the extracellular region of BTLA. In some embodiments, the animal has one or more cells expressing a chimeric BTLA having an extracellular region, a transmembrane region, and a cytoplasmic region, wherein the extracellular region comprises a sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% identical to the extracellular region of human BTLA. In some embodiments, the extracellular region of the chimeric BTLA has a sequence that has at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids that are identical to a contiguous sequence present in the extracellular region of human BTLA. In some embodiments, the animal is a mouse, and the sequence encoding the region of endogenous BTLA is exon 1, exon 2,exon 3, exon 4, exon 5, and/or exon 6 of the endogenous mouse BTLA gene. In some embodiments, the animal is heterozygous with respect to the replacement at the endogenous BTLA gene locus.In some

embodiments, the animal is homozygous with respect to the replacement at the endogenous BTLA gene locus.

In one aspect, the disclosure relates to methods for making a genetically-modified, non-human animal, including: replacing in at least one cell of the animal, at an endogenous BTLA gene locus, a sequence encoding a region of an endogenous BTLA with a sequence encoding a corresponding region of human BTLA. In some embodiments, the sequence encoding the corresponding region of human BTLA comprises exon 1, exon 2, exon 3, exon 4, and/or exon 5 of a human BTLA gene. In some embodiments, the sequence encoding the corresponding region of BTLA comprises exon 2 of a human BTLA gene, and/or a part of exon 1 and/or exon 3 of a human BTLA gene. In some embodiments, the sequence encoding the corresponding region of human BTLA encodes amino acids 34-132 of SEQ ID NO: 27. In some embodiments, the region is located within the extracellular region of BTLA. In some embodiments, the animal is a mouse, and the sequence encoding the region of the endogenous BTLA locus is exon2 of mouse BTLA gene.

In one aspect, the disclosure relates to non-human animals comprising at least one cell comprising a nucleotide sequence encoding a chimeric BTLA polypeptide, wherein the chimeric BTLA polypeptide comprises at least 50 contiguous amino acid residues that are identical to the corresponding contiguous amino acid sequence of a human BTLA, wherein the animal expresses the chimeric BTLA.In some embodiments, the chimeric BTLA polypeptide has at least 50 contiguous amino acid residues that are identical to the corresponding contiguous amino acid sequence of a human BTLA extracellular region. In some embodiments, the chimeric BTLA polypeptide comprises a sequence that is at least 90%, 95%, or 99% identical to amino acids 34-132 of SEQ ID NO: 27. In some embodiments, the nucleotide sequence is operably linked to an endogenous BTLA regulatory element of the animal.In some embodiments, the chimeric BTLA polypeptide comprises an endogenous BTLAtransmembrane region and/or an endogenous BTLA cytoplasmic region. In some embodiments, the nucleotide sequence is integrated to an endogenous BTLA gene locus of the animal.In some embodiments, the chimeric BTLA has at least one mouse BTLA activity (e.g., interacting with mouse HVEM, and inhibiting mouse T-cell immune responses) and/or at least one human BTLA activity (e.g., interacting with human HVEM, and inhibiting human T-cell immune responses).

In one aspect, the disclosure relates to methods of making a genetically-modified mouse cell that expresses a chimeric BTLA, the method including: replacing, at an endogenous mouse BTLA gene locus, a nucleotide sequence encoding a region of mouse BTLA with a nucleotide sequence encoding a corresponding region of human BTLA, thereby generating a genetically-modified mouse cell that includes a nucleotide sequence that encodes the chimeric BTLA, wherein the mouse cell expresses the chimeric BTLA.In some embodiments, the chimeric BTLA comprises an extracellular region of mouse BTLA comprising a mouse signal peptide sequence, an extracellular region of human BTLA, a transmembrane and/or a cytoplasmic region of a mouse BTLA. In some embodiments, the nucleotide sequence encoding the chimeric BTLA is operably linked to an endogenous BTLA regulatory region, e.g., promoter.

In some embodiments, the animals further comprise a sequence encoding an additional human or chimeric protein. In some embodiments, the additional human or chimeric protein is programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte Activating 3 (LAG-3), T-Cell Immunoglobulin And Mucin Domain-Containing Protein 3 (TIM-3), Programmed Cell Death 1 Ligand 1 (PD-L1), TNF Receptor Superfamily Member 9 (4-1BB), CD27, CD28, CD47, T-Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), CD27, Glucocorticoid-Induced TNFR-Related Protein (GITR), or TNF Receptor Superfamily Member 4 (TNFRSF4 or OX40). In some embodiments, the animal or mouse further comprises a sequence encoding an additional human or chimeric protein. In some embodiments, the additional human or chimeric protein is programmed cell death protein 1 (PD-1), CTLA-4, LAG-3, TIM-3, PD-L1, 4-1BB, CD27, CD28, CD47, TIGIT, CD27, GITR, or OX40.

In one aspect, the disclosure relates to methods of determining effectiveness of an anti-BTLA antibody for the treatment of cancer, including: administering the anti-BTLA antibody to the animal as described herein, wherein the animal has a tumor, and determining the inhibitory effects of the anti-BTLA antibody to the tumor. In some embodiments, the tumor comprises one or more tumor cells that express HVEM.

In some embodiments, the tumor comprises one or more cancer cells that are injected into the animal. In some embodiments, determining the inhibitory effects of the anti-BTLA antibody to the tumor involves measuring the tumor volume in the animal. In some embodiments, the tumor cells are melanoma cells, non-small cell lung carcinoma (NSCLC) cells, small cell lung cancer (SCLC) cells, bladder cancer cells, and/or prostate cancer cells (e.g., metastatic hormone-refractory prostate cancer).

In one aspect, the disclosure relates to methods of determining effectiveness of an anti-BTLA antibody for the treatment of various immune-related disorders, e.g., autoimmune diseases.

In one aspect, the disclosure relates to methods of determining effectiveness of an anti-BTLA antibody and an additional therapeutic agent for the treatment of a tumor, including administering the anti-BTLA antibody and the additional therapeutic agent to the animal as described herein, wherein the animal has a tumor, and determining the inhibitory effects on the tumor.In some embodiments, the animal further comprises a sequence encoding a human or chimeric programmed cell death protein 1 (PD-1). In some embodiments, the additional therapeutic agent is an anti-PD-1 antibody. In some embodiments, the tumor comprises one or more tumor cells that express HVEM. In some embodiments, the tumor comprises one or more tumor cells that express PD-L1 or PD-L2. In some embodiments, the tumor is caused by injection of one or more cancercellsinto the animal. In some embodiments, determining the inhibitory effects of the treatment involves measuring the tumor volume in the animal. In some embodiments, the tumor comprises melanoma cells, non-small cell lung carcinoma (NSCLC) cells, small cell lung cancer (SCLC) cells, bladder cancer cells, and/or prostate cancer cells (e.g., metastatic hormone-refractory prostate cancer cells).

In one aspect, the disclosure relates to proteins comprising an amino acid sequence, wherein the amino acid sequence is one of the following: (a) an amino acid sequence set forth in SEQ ID NO: 31; (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 31; (c) an amino acid sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 31; (d) an amino acid sequence that is different from the amino acid sequence set forth in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid; and (e) an amino acid sequence that comprises a substitution, a deletion and /or insertion of one, two, three, four, five or more amino acids to the amino acid sequence set forth in SEQ ID NO: 31.In some embodiments, provided herein are cells comprising the proteins disclosed herein. In some embodiments, provided herein are animals having the proteins disclosed herein.

In one aspect, the disclosure relates to nucleic acids comprising a nucleotide sequence, wherein the nucleotide sequence is one of the following: (a) a sequence that encodes the protein as described herein; (b) SEQ ID NO: 29; (c) SEQ ID NO: 30; (d) a sequence that is at least 90% identical to SEQ ID NO: 29 or SEQ ID NO: 30; (e) a sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 29; and (f) a sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 30.In some

embodiments, provided herein are cells comprising the nucleic acids disclosed herein.In some embodiments, provided herein are animals having the nucleic acids disclosed herein.

In one aspect, the disclosure relates to a targeting vector, including a) a DNA fragment homologous to the 5' end of a region to be altered (5' arm), which is selected from the BTLA gene genomic DNAs in the length of 100 to 10,000 nucleotides; b) a desired/donor DNA sequence encoding a donor region; and c) a second DNA fragment homologous to the 3' end of the region to be altered (3' arm), which is selected from the BTLA gene genomic DNAs in the length of 100 to 10,000 nucleotides.

In some embodiments, a) the DNA fragment homologous to the 5' end of a region to be altered (5' arm/receptor) is selected from the nucleotide sequences that have at least 90% homology to the NCBI accession number NC_000082.6; c) the DNA fragment homologous to the 3' end of the region to be altered (3' arm/receptor) is selected from the nucleotide sequences that have at least 90% homology to the NCBI accession number NC_000082.6.

In some embodiments, a) the DNA fragment homologous to the 5' end of a region to be altered (5' arm/receptor) is selected from the nucleotides from the position 45237539 to the position 45239051 of the NCBI accession number NC_000082.6; c) the DNA fragment homologous to the 3' end of the region to be altered (3' arm/receptor) is selected from the nucleotides from the position 45239358 to the position 45240854 of the NCBI accession number NC_000082.6.

In some embodiments, a length of the selected genomic nucleotide sequence is about 1.2kb, 1.5 kb or 1 kb. In some embodiments, the length is about 1513bp or 1497bp. In some embodiments, the region to be altered is exon 2 of BTLAgene.

In some embodiments, the sequence of the 5' arm is shown in SEQ ID NO: 32. In some embodiments, the sequence of the 3' arm is shown in SEQ ID NO: 38.

In some embodiments, the targeting vector further includes a selectable gene marker.

In some embodiments, the target region is derived from human. In some embodiments, the target region is a part or entirety of the nucleotide sequence of a humanized BTLA. In some embodiments, the nucleotide sequence is shown as one or

more of the first exon, the second exon, the third exon, the fourth exon, and the fifth exonof the DNA sequence of the human BTLA.

In some embodiments, the nucleotide sequence of the human BTLA encodes the human BTLA protein with the NCBI accession number NP_861445.3 (SEQ ID NO: 27).

The disclosure also relates to a cell including the targeting vector as described herein.

In another aspect, the disclosure relates to an sgRNA sequence for constructing a humanized animal model, wherein the sgRNA sequence targets the BTLA gene, the sgRNA is unique on the target sequence of the BTLA gene to be altered, and meets the sequence arrangement rule of 5'-NNN (20) -NGG3' or 5'-CCN-N (20)-3'. In some embodiments, the targeting site of the sgRNA in the mouse BTLA gene is located on the exon 2 of the mouse BTLA gene.

In another aspect, the disclosure relates to an sgRNA sequence for constructing a humanized animal model, wherein an upstream sequence thereof is shown as SEQ ID NO: 15, and a downstream sequence thereof is shown as SEQ ID NO: 17, and the sgRNA sequence recognizes a 5' targeting site.

The disclosure also relates to an sgRNA sequence for constructing a humanized animal model, wherein an upstream sequence thereof is shown as SEQ ID NO: 16, which is obtained by adding TAGG to the 5' end of SEQ ID NO: 15; a downstream sequence thereof is shown as SEQ ID NO: 18, which is obtained by adding AAAC to the 5' end of SEQ ID NO: 17, and the sgRNA sequence recognizes a 5' targeting site.

The disclosure also relates to an sgRNA sequence for constructing a humanized animal model, wherein an upstream sequence thereof is shown as SEQ ID NO: 19, and a downstream sequence thereof is shown as SEQ ID NO: 21, and the sgRNA sequence recognizes a 3' targeting site.

The disclosure further relates to an sgRNA sequence for constructing a humanized animal model, wherein an upstream sequence thereof is shown as SEQ ID NO: 20, which is obtained by adding TAGG to the 5' end of SEQ ID NO: 19; a downstream sequence thereof is shown as SEQ ID NO: 22, which is obtained by adding AAAC to the 5' end of SEQ ID NO: 21, and the sgRNA sequence recognizes a 3' targeting site.

In one aspect, the disclosure relates to a construct including the sgRNA sequence as described herein.

The disclosure also relates to a cell comprising the construct as described herein.

In another aspect, the disclosure relates to a non-human mammalian cell, comprising the targeting vector as described herein, and one or more *in vitro* transcripts of the sgRNA construct.

In some embodiments, the cell includes Cas9 mRNA or an *in vitro* transcript thereof.

In some embodiments, the genes in the cell are heterozygous. In some embodiments, the genes in the cell are homozygous.

In some embodiments, the non-human mammalian cell is a mouse cell. In some embodiments, the cell is a fertilized egg cell. In some embodiments, the cell is a germ cell.In some embodiments, the cell is a blastocyst.In some embodiments, the cell is a lymphocyte (e.g., a B-cell or a T-cell).

In another aspect, the disclosure relates to methods for establishing a BTLA gene humanized animal model. The methods include the steps of

(a) providing the cell, and preferably the cell is a fertilized egg cell;

(b) culturing the cell in a liquid culture medium;

(c) transplanting the cultured cell to the fallopian tube or uterus of the recipient female non-human mammal, allowing the cell to develop in the uterus of the female non-human mammal;

(d) identifying the germline transmission in the offspring genetically modified humanized non-human mammal of the pregnant female in step (c).

In some embodiments, the establishment of a humanized animal model of BTLA gene using a gene editing technique is based on CRISPR /Cas9.

In some embodiments, the non-human mammal is mouse. In some embodiments, the mouse is a C57BL/6 mouse. In some embodiments, the non-human mammal in step (c) is a female with false pregnancy.

The disclosure also relates to a method for establishing a genetically-modified non-human animal expressing two human or chimeric (e.g., humanized) genes. The method includes the steps of

(a) using the method for establishing a BTLA gene humanized animal model to obtain a BTLA gene genetically modified humanized mouse;

(b) mating the BTLA gene genetically modified humanized mouse obtained in step (a) with another humanized mouse, and then screening to obtain a double humanized mouse model.

In some embodiments, in step (b), the BTLA gene genetically modified humanized mouse obtained in step (a) is mated with a PD-1 humanized mouse to obtain a BTLA and PD-1 double humanized mouse model.

The disclosure also relates to non-human mammal generated through the methods as described herein.

In some embodiments, the genome thereof contains human gene(s).

In some embodiments, the non-human mammal is a rodent. In some embodiments, the non-human mammal is a mouse.

In some embodiments, the non-human mammal expresses a protein encoded by a humanized BTLA gene.

The disclosure also relates to an offspring of the non-human mammal.

In another aspect, the disclosure relates to a tumor bearing non-human mammal model, characterized in that the non-human mammal model is obtained through the method as described herein.

In some embodiments, the non-human mammal is a rodent. In some embodiments, the non-human mammal is a mouse.

The disclosure also relates to a cell or cell line, or a primary cell culture thereof derived from the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal.

The disclosure further relates to the tissue, organ or a culture thereof derived from the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal.

In another aspect, the disclosure relates to a tumor tissue derived from the non-human mammal or an offspring thereof when it bears a tumor, or the tumor bearing non-human mammal.

In one aspect, the disclosure relates to a BTLA amino acid sequence of a humanized mouse, wherein the amino acid sequence is selected from the group consisting of:

a) an amino acid sequence shown in SEQ ID NO: 31;

b) an amino acid sequence having a homology of at least 90% with the amino acid sequence shown in SEQ ID NO: 31;

c) an amino acid sequence encoded by a nucleic acid sequence, wherein the nucleic acid sequence is able to hybridize to a nucleotide sequence encoding the amino acid shown in SEQ ID NO: 31 under a low stringency condition;

d) an amino acid sequence having a homology of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the amino acid sequence shown in SEQ ID NO: 31;

e) an amino acid sequence that is different from the amino acid sequence shown in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or no more than 1 amino acid; or

f) an amino acid sequence that comprises a substitution, a deletion and /or insertion of one or more amino acids to the amino acid sequence shown in SEQ ID NO: 31.

The disclosure also relates to a BTLA DNA sequence of a humanized mouse, wherein the DNA sequence is selected from the group consisting of:

a) a DNA sequence that encodes the BTLA amino acid sequence of a humanized mouse;

b) a DNA sequence that is set forthinSEQ ID NO: 35;

c) a DNA sequence having a coding DNA sequence (CDS) as shown in SEQ ID NO: 29;

d) a DNA sequence that is able to hybridize to the nucleotide sequence as shown in SEQ ID NO: 35 or SEQ ID NO: 29 under a low stringency condition;

e) a DNA sequence that has a homology of at least 90% with the nucleotide sequence as shown in SEQ ID NO: 29 or SEQ ID NO: 30;

f) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence has a homology of at least 90% with the amino acid sequence shown in SEQ ID NO: 31;

g) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence has a homology of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99% with the amino acid sequence shown in SEQ ID NO: 31;

h) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence is different from the amino acid sequence shown in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or no more than 1 amino acid; and/or

i) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence comprises a substitution, a deletion and /or insertion of 1, 2, 3, 4, 5, 6, 7, 8, 9, or more amino acids to the amino acid sequence shown in SEQ ID NO: 31.

j) and optimized SEQ ID NO: 35.

The disclosure further relates to a BTLA genomic DNA sequence of a humanized mouse, a DNA sequence obtained by a reverse transcription of the mRNA obtained by transcription thereof is consistent with or complementary to the DNA sequence; a construct expressing the amino acid sequence thereof; a cell comprising the construct thereof; a tissue comprising the cell thereof.

The disclosure further relates to the use of the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal, the animal model generated through the method as described herein in the development of a product related to an immunization processes of human cells, the manufacture of a human antibody, or the model system for a research in pharmacology, immunology, microbiology and medicine.

The disclosure also relates to the use of the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal, the animal model generated through the method as described herein in the production and utilization of an animal experimental disease model of an immunization processes involving human cells, the study on a pathogen, or the development of a new diagnostic strategy and /or a therapeutic strategy.

The disclosure further relates to the use of the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal, the animal model generated

through the methods as described herein, in the screening, verifying, evaluating or studying the BTLA gene function, human BTLA antibodies, the drugs or efficacies for human BTLA targeting sites, and the drugs for immune-related diseases and antitumor drugs.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs.Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also be used.The materials, methods, and examples are illustrative only and not intended to be limiting.All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their entirety.In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

DESCRIPTION OF DRAWINGS

FIG. 1A is a graph showing the 5' terminal target site sgRNA activity test results (sgRNA1-sgRNA8) (Con is a negative control; and PC is a positive control).

FIG. 1B is a graph showing 3' terminal target site sgRNA activity test results (sgRNA9-sgRNA14) (Con is a negative control; and PC is a positive control).

FIG. 2 is a schematic diagram showing pT7-sgRNA plasmid map.

FIG. 3A is a schematic diagram showing comparison of human and mouse BTLA genes.

FIG. 3B is a schematic diagram showing humanized BTLA mouse gene map.

FIG. 3C is a schematic diagram showing mouse BTLA gene targeting strategy.

FIG. 4A shows pClon-4G-BTLA plasmid digestion result (M is the Marker, ck is undigested plasmid.)

FIG. 4B shows the fragment sizes for the Marker.

FIG. 5 shows PCR identification result of samples collected from tails of F0 generation mice (M is the Marker; WT is wild type; mice labeled with No. 1 and 2 are positive).

FIG. 6 shows PCR identification result of samples collected from tails of F1 generation mice (M is the Marker; WT is wild type; + is positive control; mice labeled with F1-1 to F1-6 are all positive).

FIG. 7A shows Southern blot results for F1 generation mice by P1 probe (WT is wild type).

FIG. 7B shows Southern blot results for F1-4 mouse by P2 probe (WT is wild type); the results show that the mouse labeled with F1-4 has no random insertion.

FIGS. 8A-8F are graphs of flow cytometry analysis results for C57BL/6 mice and BTLA humanized mice. The anti-mouse CD3 antibody was used to stimulate the T cells in the spleen, and then anti-mouse BTLA antibodies and anti-mTCRβ antibodies (FIGS. 8A-8C), or anti-human BTLA antibodies and anti-mTCRβ antibodies (FIGS. 8D-8F),were used to label cells. Compared to the control group (FIGS. 8A and 8D), the cells with the expression of human BTLA protein can be detected in the spleen of BTLA humanized F1 hybrids (FIG. 8F); whereas in the spleen of C57BL/6 mice, no cells expressing human BTLA protein were detected (FIG. 8E).

FIG. 9 shows RT-PCR detection results, wherein +/+ is wild type C57BL/6 mouse; H/+ is F1 generation hBTLA heterozygous mouse; and GAPDH is an internal control.

FIGS. 10A-10F are graphs of flow cytometry analysis results for C57BL/6 mice and hBTLA homozygous mice. The anti-mouse CD3 antibody was used to stimulate the T cells in the spleen, and then anti-mouse BTLA antibodies (mBTLA PE) and anti-mCD19 antibodies (mCD19 FITC) (FIGS. 10A-10C), or anti-human BTLA antibodies (hBTLA APC) and anti-mCD19 antibodies (mCD19 FITC) (FIGS. 10D-10F), were used to label T cells. Mouse BTLA protein can be detected in the spleen of C57BL/6 mice (FIGS. 10A and 10B). Human BTLA protein can be detected in the spleen of hBTLA homozygous mice (FIG. 10F).

FIG. 11 shows RT-PCR detection results, wherein +/+ is wild type C57BL/6 mouse; H/H is B-hBTLA homozygous mouse; and GAPDH is an internal control.

FIG. 12 shows PCR identification results for BTLA gene knockout mice, wherein WT is wild type, M is the maker, + is the positive control, the mice with No. 1-6 are BTLA knockout mice.

FIG. 13.Mouse colon cancer cells MC38 were injected into B-hBTLA mice and antitumor efficacy studies were performed for 6 anti-human BTLA antibodies (AB1, AB2, AB3, AB4, AB5, AB6, 10mg/kg). There was no significant difference in average weight gain between the G1 control group and the G2-G7 treatment groups.

FIG. 14.Mouse colon cancer cells MC38 were injected into B-hBTLA mice and antitumor efficacy studies were performed for 6 anti-human BTLA antibodies (AB1, AB2, AB3, AB4, AB5, AB6, 10mg/kg). There was no significant difference in body weight change percentage among different groups.

FIG. 15. Mouse colon cancer cells MC38 were injected into B-hBTLA mice and antitumor efficacy studies were performed for 6 anti-human BTLA antibody (AB1, AB2, AB3, AB4, AB5, AB6, 10mg/kg). The average volumes of tumors in the G3-G7 treatment groupswere smaller than the G1 control group, and the differences were significant.

FIGS. 16A-16B. Mouse tail PCR identification result, where + is hBTLA homozygous positive control, - is wildtype negative control. The mice numbered 3017-3032 are homozygous for humanized BTLA gene.

FIGS. 16C-16D. Mouse tail PCR identification result, where WT is wildtype, -/- is humanized PD-1 homozygous mouse, +/- is humanized PD-1 heterozygous mouse. The mice numbered 3017-3032 are homozygous for humanized PD-1 gene

FIGS. 17A- 17F show flow cytometry analysis results for C57BL/6 mice and double humanized BTLA/PD-1 homozygous mice. Anti-mouse CD3 antibody was used to stimulate T cell activation in the spleens of the mice, and then the mouse BTLA antibody (mBTLAPE)and anti-mCD19 antibodies (mCD19 FITC)(FIGS. 17A, 17B, 17C), or human BTLA antibody hBTLAAPCand anti-mCD19 antibodies (mCD19 FITC)(FIGS. 17D, 17E, 17F), were used to label T cell surface proteins. The result show that the cells expressing humanized BTLA proteins were detected in the spleens of double humanized BTLA /PD-1 mice, while no cells expressing humanized BTLA protein were detected in the spleen of C57BL/6 control mice.

FIGS. 18A- 18F show flow cytometry analysis results for C57BL/6 mice and double humanized BTLA/PD-1 homozygous mice. Anti-mouse CD3 antibody was used to stimulate T cell activation in the spleens of the mice, and then the mouse PD-1 antibody (mPD-1 PE) and mouse T cell surface antibody mTcRβ (FIGS. 18A, 18B, 18C), or human PD-1 antibody hPD-1 FITC and mouse T cell surface antibody mTcRβ (FIGS. 18D, 18E, 18F), were used to label T cell proteins. The result show that the cells expressing humanized PD-1 proteins were detected in the spleens of double humanized BTLA /PD-1 mice, while no cells expressing humanized PD-1 protein were detected in the spleen of C57BL/6 control mice.

FIG. 19 shows RT-PCR detection results for mBTLA or humanized BTLA (hBTLA), wherein +/+ is wild type C57BL/6 mouse; H/H is double humanized BTLA/PD-1 homozygous mice; and GAPDH is an internal control.

FIG. 20 shows RT-PCR detection results for mPD-1 or humanized PD-1 (hPD-1), wherein +/+ is wild type C57BL/6 mouse; H/H is double humanized BTLA/PD-1 homozygous mice; and GAPDH is an internal control.

FIG. 21 is a schematic diagram of the targeting strategy for embryonic stem cells.

FIG. 22 shows the alignment between mouse BTLA amino acid sequence (NP_001032808.2; SEQ ID NO:25) and human BTLA amino acid sequence (NP_861445.3; SEQ ID NO:27) by NCBI Basic Local Alignment Search Tool (BLAST).

DETAILED DESCRIPTION

This disclosure relates to transgenic non-human animal with human or chimeric (e.g., humanized) B- And T-Lymphocyte-Associated Protein (BTLA or CD272), and methods of use thereof.

BTLA is a T cell inhibitory receptor. It is expressed on the cell surface of B cells, T cells, and macrophages. BTLA expression is induced during activation of T cells and is expressed on developing TH_1 and TH_2 cells. Expression of BTLA is subsequently lost on highly differentiated TH_2 cells but remains on TH_1 cells. Results show that coligation of BTLA partially inhibitsCD3-induced secretion of IL-2 and that BTLA-deficient T cells haveincreased proliferation to antigen presented by dendritic cells (DCs),suggesting that BTLA exerts an inhibitory rather than activating influenceon T cells(Watanabe, Norihiko,

et al. "BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1." Nature immunology 4.7 (2003): 670).

BTLA is similar to cytotoxic T-lymphocyte-associated protein 4(CTLA-4)andprogrammed death 1 (PD-1), two other inhibitory receptors expressed on T lymphocytes.Like PD-1 and CTLA-4, BTLA interacts with a B7 homolog, B7H4. However, BTLA also inhibits T-Cells via interaction with tumor necrosis family receptors (TNF-R). BTLA is a ligand for tumor necrosis factor (receptor) superfamily, member 14 (TNFRSF14), also known as herpes virus entry mediator (HVEM). BTLA-HVEM complexes negatively regulate T-cell immune responses.The function and the structure of BTLA is described, e.g., in Watanabe, et al. "BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1," Nature immunology 4.7 (2003): 670;Steinberg et al. "BTLA interaction with HVEM expressed on CD8+ T cells promotes survival and memory generation in response to a bacterial infection," PLoS One 8.10 (2013): e77992; Murphyet al., "Balancing co-stimulation and inhibition with BTLA and HVEM," Nature reviews. Immunology 6.9 (2006): 671; each of which is incorporated by reference in its entirety.

As BTLA is involved in T cell inhibitory pathway, it thus can be expected that the BTLA antibody has great application values, e.g., as a tumor immunotherapy or a treatment for autoimmune disease (e.g., systemic lupus erythematosus, and Sjögren's syndrome). In order to make the animal experiments more effective and more relevant, the present disclosure provides humanized BTLA genetically modified animal models and methods of establishing such animal models.

Experimental animal models are an indispensable research tool for studying the etiology, pathogenesis of the disease, as well as the development of prevention and control techniques and therapeutic drugs for the disease. Common experimental animals include mice, rats, guinea pigs, hamsters, rabbits, dogs, monkeys, pigs, fish and so on. However, there are many differences between human and animal genes and protein sequences, and many human proteins cannot bind to the animal's homologous proteins to produce biological activity, leading to that the results of many clinical trials do not match the results obtained from animal experiments. A large number of clinical studies are in urgent need of better animal models. With the continuous development and maturation of

genetic engineering technologies, the use of human cells or genes to replace or substitute an animal's endogenous similar cells or genes to establish a biological system or disease model closer to human, and establish the humanized experimental animal models (humanized animal model) has provided an important tool for new clinical approaches or means. In this context, the genetically engineered animal model, that is, the use of genetic manipulation techniques, the use of human normal or mutant genes to replace animal homologous genes, can be used to establish the genetically modified animal models that are closer to human gene systems. The humanized animal models have various important applications. For example, due to the presence of human or humanized genes, the animals can express or express in part of the proteins with human functions, so as to greatly reduce the differences in clinical trials between humans and animals, and provide the possibility of drug screening at animal levels.

Unless otherwise specified, the practice of the methods described herein can take advantage of the techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA and immunology. These techniques are explained in detail in the following literature, for examples: Molecular Cloning A Laboratory Manual, 2nd Ed., ed. By Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press: 1989); DNA Cloning, Volumes I and II (D. N. Glovered., 1985); Oligonucleotide Synthesis (M. J. Gaited., 1984);Mullisetal U. S. Pat. No.4, 683, 195; Nucleic Acid Hybridization (B. D. Hames& S. J. Higginseds. 1984); Transcription And Translation (B. D. Hames& S. J. Higginseds. 1984); Culture Of Animal Cell (R. I. Freshney, Alan R. Liss, Inc., 1987); Immobilized Cells And Enzymes (IRL Press, 1986); B. Perbal, A Practical Guide To Molecular Cloning (1984), the series, Methods In ENZYMOLOGY (J. Abelson and M. Simon, eds.-in-chief, Academic Press, Inc., New York), specifically, Vols.154 and 155 (Wuetal. eds.) and Vol.185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Caloseds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Hand book Of Experimental Immunology, Volumes V (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor

Laboratory Press, Cold Spring Harbor, N. Y., 1986), each of which is incorporated herein in its entirety by reference.

BTLA (B And T Lymphocyte Associated or CD272)

B And T Lymphocyte Associated (BTLA), also known as CD272 or B- And T-Lymphocyte Attenuator, is an Ig super family protein with an intermediate type Ig fold in the ectodomain and an ITIM inhibitory signaling domain in the cytosol. BTLA is a ligand for tumor necrosis factor (receptor) superfamily, member 14 (TNFRSF14), also known as herpes virus entry mediator (HVEM). Engagement of BTLA by HVEM, induces tyrosine phosphorylation of the ITIM motifs in the cytoplasmic tail of BTLA, allowing the recruitment of the phosphatases SHP-1 and SHP-2, which attenuate signaling.

BTLA and its herpesvirus entry mediator (HVEM) are the only pair of molecules that have been found so far to connect the Ig superfamily proteins and TNFR family proteins. BTLA / HVEM is quite unique because BTLA mainly acts as a negative feedback regulator, which attenuates the immune response of T cells after HVEM binds to BTLA. In addition, HVEM also binds to LIGHT (TNF Superfamily Member 14; TNFSF14) and plays as a co-stimulatory actor, promoting T cells, B cell proliferation and Ig production. In addition, HVEM may bind to BTLA, and LIGHT or LTα in the same time, and form a trimer. Recent studies have shown that BTLA signaling is involved in preventing autoimmune diseases, reducing inflammation, maintaining peripheral immune tolerance, and inhibiting the immune response. BTLA inhibitors can also enhance TCR signaling and restore T cell function. BTLA can also function as an activating ligand for HVEM promoting NF-κB activation (Watanabe N, Gavrieli M, Sedy JR, et al. BTLA is a lymphocyte inhibitory receptor with similarities to CTLA-4 and PD-1. Nature Immunology. 2003;4(7):670–679), which can promote cell survival.

In human genomes, BTLA gene locus has fiveexons, exon 1, exon 2, exon 3,exon 4, and exon 5 (FIG. 3A). The BTLA protein also has an extracellular region, a transmembrane region, and a cytoplasmic region, and the signal peptide is located at the extracellular region of BTLA.The nucleotide sequence for human BTLA mRNA is NM_181780.3 (SEQ ID NO: 26), and the amino acid sequence for human BTLA is

NP_861445.3 (SEQ ID NO: 27). The location for each exon and each region in human BTLA nucleotide sequence and amino acid sequence is listed below:

Table 1

Human BTLA (approximate location)	**NM_181780.3 3216 bp (SEQ ID NO: 26)**	**NP_861445.3 289 aa (SEQ ID NO: 27)**
Exon 1	1-291	1-29
Exon 2	292-606	30-134
Exon 3	607-750	135-182
Exon 4	751-797	183-198
Exon 5	798-3215	199-289
Signal peptide	204-293	1-30
Extracellular region (excluding signal peptide region)	294-674	31 – 157
Transmembrane region	675-737	158 – 178
Cytoplasmic region	738-1070	179 – 289
Donor region in Example	303-599	34-132

Similarly, in mice, BTLA gene locus has sixexons, exon 1, exon 2, exon 3, exon 4, exon 5, and exon 6 (FIG. 3A). The BTLA protein also has an extracellular region, a transmembrane region, and a cytoplasmic region, and the signal peptide is located at the extracellular region of BTLA.The nucleotide sequence for mouse BTLA cDNA is NM_001037719.2 (SEQ ID NO: 24), the amino acid sequence for mouse BTLA is NP_001032808.2 (SEQ ID NO: 25). The location for each exon and each region in the mouse BTLA nucleotide sequence and amino acid sequence is listed below:

Table 2

Mouse BTLA (approximate location)	**NM_001037719.2 3235 bp (SEQ ID NO: 24)**	**NP_001032808.2 306 aa (SEQ ID NO: 25)**
Exon 1	1-125	1-36
Exon 2	126-446	37-143
Exon 3	447-482	144-155
Exon 4	483-641	156-208
Exon 5	642-688	209-224
Exon 6	689-3235	225-306
Signal peptide	17-103	1-29
Extracellular region (excluding signal peptide region)	104-565	30-183

Transmembrane region	566-628	184 – 204
Cytoplasmic region	629-934	205-306
Replaced region in Example	134-439	40-141

The mouse BTLA gene (Gene ID: 208154) is located in Chromosome 16 of the mouse genome, which is located from 45,223,545 to 45,252,895 of NC_000082.6 (GRCm38.p4 (GCF_000001635.24)). The 5'-UTR is from 45,224,337 to 45,224,352, exon 1 is from 45,224,353to 45,224,461, the first intron is from 45,224,462 to 45,239,043, exon 2 is from 45,239,044 to 45,239,364,the second intron is from 45,239,365 to 45,242,705, exon 3 is from 45,242,706 to 45,242,741, the third intron is from 45,242,742 to 45,244,152, exon 4 is from 45,244,153 to 45,244,311, the fourth intron is from 45,244,312 to 45,246,250, exon 5 is from 45,246,251 to 45,246,297, the fifth intron is from 45,246,298 to 45,250,348, exon 6 is from 45,250,349 to 45,250,597, the 3'-UTR is from 45,250,598 to45,252,895 of NC_000082.6, based on transcriptNM_001037719.2. All relevant information formouseBTLAlocus can be found in the NCBI website with Gene ID: 208154, which is incorporated by reference herein in its entirety.

FIG. 22 shows the alignment between mouse BTLA amino acid sequence (NP_001032808.2; SEQ ID NO: 25) and human BTLA amino acid sequence (NP_861445.3; SEQ ID NO: 27). Thus, the corresponding amino acid residue or region between human and mouse BTLAcan also be found in FIG. 22.

BTLA genes, proteins, and locus of the other species are also known in the art. For example, the gene ID for BTLA in *Rattusnorvegicus*is 407756, the gene ID for BTLA in *Macacamulatta*(Rhesus monkey) is 708202, the gene ID for BTLA in *Susscrofa*(pig)is100626925. The relevant information for these genes (e.g., intron sequences, exon sequences, amino acid residues of these proteins) can be found, e.g., in NCBI database.

The present disclosure provides human or chimeric (e.g., humanized) BTLA nucleotide sequence and/or amino acid sequences. In some embodiments, the entire sequence of mouse exon 1, exon 2, exon 3, exon 4,exon 5, exon 6, signal peptide, extracellular region, transmembrane region, and/or cytoplasmic region are replaced by the corresponding human sequence. In some embodiments, a "region" or "portion" of

mouse exon 1, exon 2, exon 3, exon 4, exon 5, exon 6, signal peptide, extracellular region, transmembrane region, and/or cytoplasmic region are replaced by the corresponding human sequence. The term “region” or “portion” can refer to at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, or 400 nucleotides, or at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, or 150 amino acid residues. In some embodiments, the “region” or “portion” can be at least 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 99% identical to exon 1, exon 2, exon 3, exon 4,exon 5, exon 6, signal peptide, extracellular region, transmembrane region, or cytoplasmic region. In some embodiments, a region, a portion, or the entire sequence of mouse exon1, exon 2, exon 3, exon 4, exon 5 and/or exon 6 (e.g., exon 2) are replaced by the human exon1, exon 2, exon 3, exon 4, and/or exon 5 (e.g., exon 2) sequence.

In some embodiments, the present disclosure also provides a chimeric (e.g., humanized) BTLA nucleotide sequence and/or amino acid sequences, wherein in some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the sequence are identical to or derived from mouse BTLA mRNA sequence (e.g., SEQ ID NO: 24), or mouse BTLA amino acid sequence (e.g., SEQ ID NO: 25); and in some embodiments, at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% of the sequence are identical to or derived from human BTLA mRNA sequence (e.g., SEQ ID NO: 26), or human BTLA amino acid sequence (e.g., SEQ ID NO: 27).

In some embodiments, the sequence encoding amino acids 40-141 of mouse BTLA (SEQ ID NO: 25) is replaced. In some embodiments, the sequence is replaced by a sequence encoding a corresponding region of human BTLA (e.g., amino acids 34-132 of human BTLA (SEQ ID NO: 27).

In some embodiments, the nucleic acids as described herein are operably linked to a promotor or regulatory element, e.g., an endogenous mouse BTLA promotor, an inducible promoter, an enhancer, and/or mouse or human regulatory elements.

In some embodiments, the nucleic acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides, e.g., contiguous or non-contiguous nucleotides) that are different from a portion of or the entire mouse BTLA nucleotide sequence (e.g., NM_001037719.2 (SEQ ID NO: 24)).

In some embodiments, the nucleic acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides, e.g., contiguous or non-contiguous nucleotides) that is the same as a portion of or the entire mouse BTLA nucleotide sequence (e.g., NM_001037719.2 (SEQ ID NO: 24)).

In some embodiments, the nucleic acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides, e.g., contiguous or non-contiguous nucleotides) that is different from a portion of or the entire human BTLA nucleotide sequence (e.g., NM_181780.3 (SEQ ID NO: 26)).

In some embodiments, the nucleic acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 nucleotides, e.g., contiguous or non-contiguous nucleotides) that is the same as a portion of or the entire human BTLA nucleotide sequence (e.g., NM_181780.3 (SEQ ID NO: 26)).

In some embodiments, the amino acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid residues, e.g., contiguous or non-contiguous amino acid residues) that is different from a portion of or the entire mouse BTLA amino acid sequence (e.g., NP_001032808.2 (SEQ ID NO: 25)).

In some embodiments, the amino acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid residues, e.g., contiguous or non-contiguous amino acid residues) that is the same as a portion of or the entire mouse BTLA amino acid sequence (e.g., NP_001032808.2 (SEQ ID NO: 25)).

In some embodiments, the amino acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid residues, e.g., contiguous or non-contiguous amino acid residues) that is different from a portion of or the entire human BTLA amino acid sequence (e.g., NP_861445.3 (SEQ ID NO: 27)).

In some embodiments, the amino acid sequence has at least a portion (e.g., at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acid residues, e.g., contiguous or non-contiguous amino acid residues) that is the same as a portion of or the entire human BTLA amino acid sequence (e.g., NP_861445.3 (SEQ ID NO: 27)).

The present disclosure also provides a humanized BTLA mouse amino acid sequence, wherein the amino acid sequence is selected from the group consisting of:

a) an amino acid sequence shown in SEQ ID NO: 31;

b) an amino acid sequence having a homology of at least 90% with or at least 90% identical to the amino acid sequence shown in SEQ ID NO: 31;

c) an amino acid sequence encoded by a nucleic acid sequence, wherein the nucleic acid sequence is able to hybridize to a nucleotide sequence encoding the amino acid shown in SEQ ID NO: 31 under a low stringency condition;

d) an amino acid sequence having a homology of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%, or at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence shown in SEQ ID NO: 31;

e) an amino acid sequence that is different from the amino acid sequence shown in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or no more than 1 amino acid; or

f) an amino acid sequence that comprises a substitution, a deletion and /or insertion of one or more amino acids to the amino acid sequence shown in SEQ ID NO: 31.

The present disclosure also relates to a BTLA DNA sequence, wherein the DNA sequence can be selected from the group consisting of:

a) a DNA sequence as shown in SEQ ID NO: 29, or a DNA sequence encoding a homologous BTLA amino acid sequence of a humanized mouse;

b) a DNA sequence that is shown in SEQ ID NO: 30;

c) a DNA sequence that is able to hybridize to the nucleotide sequence as shown in SEQ ID NO: 29 or SEQ ID NO: 30 under a low stringency condition;

d) a DNA sequence that has a homology of at least 90% or at least 90% identical to the nucleotide sequence as shown in SEQ ID NO: 29 or SEQ ID NO: 30;

e) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence has a homology of at least 90% with or at least 90% identical to the amino acidsequence shown in SEQ ID NO: 31;

f) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence has a homology of at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% with, or at least 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to the amino acid sequence shown in SEQ ID NO: 31;

g) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence is different from the amino acid sequence shown in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or no more than 1 amino acid; and/or

h) a DNA sequence that encodes an amino acid sequence, wherein the amino acid sequence comprises a substitution, a deletion and /or insertion of one or more amino acids to the amino acid sequence shown in SEQ ID NO: 31.

The present disclosure further relates to a BTLA genomic DNA sequence of a humanized mouse. The DNA sequence is obtained by a reverse transcription of the mRNA obtained by transcription thereof is consistent with or complementary to the DNA sequence homologous to the sequence shown in SEQ ID NO: 29 or SEQ ID NO: 30.

The disclosure also provides an amino acid sequence that has a homology of at least 90% with, or at least 90% identical to the sequence shown in SEQ ID NO: 31, and has protein activity. In some embodiments, the homology with the sequence shown in SEQ ID NO: 31 is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99%. In some embodiments, the foregoing homology is at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, or at least about 59%.

In some embodiments, the percentage identity with the sequence shown in SEQ ID NO: 31 is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least

99%. In some embodiments, the foregoing percentage identity is at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, or at least about 59%.

The disclosure also provides a nucleotide sequence that has a homology of at least 90%, or at least 90% identical to the sequence shown in SEQ ID NO: 30, and encodes a polypeptide that has protein activity. In some embodiments, the homology with the sequence shown in SEQ ID NO: 30 is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99%. In some embodiments, the foregoing homology is at least bout 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, or at least about 59%.

In some embodiments, the percentage identity with the sequence shown in SEQ ID NO: 30 is at least about 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or at least 99%. In some embodiments, the foregoing percentage identity is at least about 50%, 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, or at least about 59%.

The disclosure also provides a nucleic acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any nucleotide sequence as described herein, and an amino acid sequence that is at least 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% identical to any amino acid sequence as described herein.In some embodiments, the disclosure relates to nucleotide sequences encoding any peptides that are described herein, or any amino acid sequences that are encoded by any nucleotide sequences as described herein. In some embodiments, the nucleic acid sequence is less than 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 150, 200, 250, 300, 350, 400, or 500 nucleotides. In some embodiments, the amino acid sequence is less than 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, or 150 amino acid residues.

In some embodiments, the amino acid sequence (i) comprises an amino acid sequence; or (ii) consists of an amino acid sequence, wherein the amino acid sequence is any one of the sequences as described herein.

In some embodiments, the nucleic acid sequence (i) comprises a nucleic acid sequence; or (ii) consists of a nucleic acid sequence, wherein the nucleic acid sequence is any one of the sequences as described herein.

To determine the percent identity of two amino acid sequences, or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes).The length of a reference sequence aligned for comparison purposes is at least 80% of the length of the reference sequence, and in some embodiments is at least 90%, 95%, or 100%.The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared.When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position.The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences. For purposes of the present disclosure, the comparison of sequences and determination of percent identity between two sequences can be accomplished using a Blossum 62 scoring matrix with a gap penalty of 12, a gap extend penalty of 4, and a frameshift gap penalty of 5.

The term "percent homology" is often used to mean "sequence similarity." The percentage of identical residues (percent identity) and the percentage of residues conserved with similar physicochemical properties (percent similarity), e.g. leucine and isoleucine, are both used to "quantify the homology". Residues conserved with similar physicochemical properties are well known in the art. The percent homology, in many cases, is higher than the percent identity.

Cells, tissues, and animals (e.g., mouse) are also provided that comprise the nucleotide sequences as described herein, as well as cells, tissues, and animals (e.g., mouse) that express human or chimeric (e.g., humanized)BTLA from an endogenous non-human BTLA locus.

Genetically modified animals

As used herein, the term “genetically-modified non-human animal” refers to a non-human animal having exogenous DNA in at least one chromosome of the animal’s

genome.In some embodiments, at least one or more cells, e.g., at least 1%, 2%, 3%, 4%, 5%, 10%, 20%, 30%, 40%, 50% of cells of the genetically-modified non-human animal have the exogenous DNA in its genome. The cell having exogenous DNA can be various kinds of cells, e.g., an endogenous cell, a somatic cell, an immune cell, a T cell, a B cell, a germ cell, a blastocyst, or an endogenous tumor cell. In some embodiments, genetically-modified non-human animals are provided that comprise a modified endogenous BTLA locus that comprises an exogenous sequence (e.g., a human sequence), e.g., a replacement of one or more non-human sequences with one or more human sequences. The animals are generally able to pass the modification to progeny, i.e., through germline transmission.

As used herein, the term "chimeric gene" or "chimeric nucleic acid" refers to a gene or a nucleic acid, wherein two or more portions of the gene or the nucleic acid are from different species, or at least one of the sequences of the gene or the nucleic acid does not correspond to the wildtype nucleic acid in the animal. In some embodiments, the chimeric gene or chimeric nucleic acid has at least one portion of the sequence that is derived from two or more different sources, e.g., sequences encoding different proteins or sequences encoding the same (or homologous) protein of two or more different species. In some embodiments, the chimeric gene or the chimeric nucleic acid is a humanized gene or humanized nucleic acid.

As used herein, the term "chimeric protein" or "chimeric polypeptide" refers to a protein or a polypeptide, wherein two or more portions of the protein or the polypeptide are from different species, or at least one of the sequences of the protein or the polypeptide does not correspond to wildtype amino acid sequence in the animal. In some embodiments, the chimeric protein or the chimeric polypeptide has at least one portion of the sequence that is derived from two or more different sources, e.g., same (or homologous) proteins of different species. In some embodiments, the chimeric protein or the chimeric polypeptide is a humanized protein or a humanized polypeptide.

In some embodiments, the chimeric gene or the chimeric nucleic acid is a humanized BTLA gene or a humanized BTLA nucleic acid. In some embodiments, at least one or more portions of the gene or the nucleic acid is from the human BTLA gene, at least one or more portions of the gene or the nucleic acid is from a non-human

BTLAgene. In some embodiments, the gene or the nucleic acid comprises a sequence that encodes a BTLA protein. The encoded BTLA protein is functional or has at least one activity of the human BTLA protein or the non-human BTLA protein, e.g., binding to human or non-human HVEM and/or B7-H4 (VTCN1), regulating immune response, promoting NF-κB activation, and/or promoting cell (e.g., T cell) survival.

In some embodiments, the chimeric protein or the chimeric polypeptide is a humanized BTLA protein or a humanized BTLA polypeptide. In some embodiments, at least one or more portions of the amino acid sequence of the protein or the polypeptide is from a human BTLA protein, and at least one or more portions of the amino acid sequence of the protein or the polypeptide is from a non-human BTLA protein. The humanized BTLA protein or the humanized BTLA polypeptide is functional or has at least one activity of the human BTLA protein or the non-human BTLA protein

The genetically modified non-human animal can be various animals, e.g., a mouse, rat, rabbit, pig, bovine (e.g., cow, bull, buffalo), deer, sheep, goat, chicken, cat, dog, ferret, primate (e.g., marmoset, rhesus monkey). For the non-human animals where suitable genetically modifiable ES cells are not readily available, other methods are employed to make a non-human animal comprising the genetic modification. Such methods include, e.g., modifying a non-ES cell genome (e.g., a fibroblast or an induced pluripotent cell) and employing nuclear transfer to transfer the modified genome to a suitable cell, e.g., an oocyte, and gestating the modified cell (e.g., the modified oocyte) in a non-human animal under suitable conditions to form an embryo. These methods are known in the art, and are described, e.g., in A. Nagy, et al., "Manipulating the Mouse Embryo: A Laboratory Manual (Third Edition)," Cold Spring Harbor Laboratory Press, 2003, which is incorporated by reference herein in its entirety.

In one aspect, the animal is a mammal, e.g., of the superfamily *Dipodoidea* or *Muroidea*. In some embodiments, the genetically modified animal is a rodent.The rodent can be selected from a mouse, a rat, and a hamster. In some embodiment, the rodent is selected from the superfamily *Muroidea*. In some embodiments, the genetically modified animal is from a family selected from *Calomyscidae* (e.g., mouse-like hamsters), *Cricetidae* (e.g., hamster, New World rats and mice, voles), *Muridae* (true mice and rats, gerbils, spiny mice, crested rats), *Nesomyidae*(climbing mice, rock mice, with-tailed rats,

Malagasy rats and mice), *Platacanthomyidae*(e.g., spiny dormice), and *Spalacidae* (e.g., mole rates, bamboo rats, and zokors). In some embodiments, the genetically modified rodent is selected from a true mouse or rat (family *Muridae*), a gerbil, a spiny mouse, and a crested rat. In one embodiment, the non-human animal is a mouse.

In some embodiments, the animal is a mouse of a C57BL strain selected from C57BL/A, C57BL/An, C57BL/GrFa, C57BL/KaLwN, C57BL/6, C57BL/6J, C57BL/6ByJ, C57BL/6NJ, C57BL/10, C57BL/10ScSn, C57BL/10Cr, and C57BL/Ola. In some embodiments, the mouse is a 129 strain selected from the group consisting of a strain that is 129P1, 129P2, 129P3, 129X1, 129S1 (e.g., 129S1/SV, 129S1/SvIm), 129S2, 129S4, 129S5, 129S9/SvEvH, 129S6 (129/SvEvTac), 129S7, 129S8, 129T1, 129T2. These mice are described, e.g., in Festing et al., Revised nomenclature for strain 129 mice, Mammalian Genome 10:836 (1999); Auerbach et al., Establishment and Chimera Analysis of 129/SvEv- and C57BL/6-Derived Mouse Embryonic Stem Cell Lines (2000), both of which are incorporated herein by reference in the entirety. In some embodiments, the genetically modified mouse is a mix of the 129 strain and the C57BL/6 strain. In some embodiments, the mouse is a mix of the 129 strains, or a mix of the BL/6 strains. In some embodiment, the mouse is a BALB strain, e.g., BALB/c strain. In some embodiments, the mouse is a mix of a BALB strain and another strain. In some embodiments, the mouse is from a hybrid line (e.g., 50% BALB/c-50% 12954/Sv; or 50% C57BL/6-50% 129).

In some embodiments, the animal is a rat. The rat can be selected from a Wistar rat, an LEA strain, a Sprague Dawley strain, a Fischer strain, F344, F6, and Dark Agouti. In some embodiments, the rat strain is a mix of two or more strains selected from the group consisting of Wistar, LEA, Sprague Dawley, Fischer, F344, F6, and Dark Agouti.

The animal can have one or more other genetic modifications, and/or other modifications, that are suitable for the particular purpose for which the humanized BTLA animal is made. For example, suitable mice for maintaining a xenograft (e.g., a human cancer or tumor), can have one or more modifications that compromise, inactivate, or destroy the immune system of the non-human animal in whole or in part. Compromise, inactivation, or destruction of the immune system of the non-human animal can include, for example, destruction of hematopoietic cells and/or immune cells by chemical means

(e.g., administering a toxin), physical means (e.g., irradiating the animal), and/or genetic modification (e.g., knocking out one or more genes). Non-limiting examples of such mice include, e.g., NOD mice, SCID mice, NOD/SCID mice, IL2Rγ knockout mice, NOD/SCID/γcnull mice (Ito, M. et al., NOD/SCID/ γcnull mouse: an excellent recipient mouse model for engraftment of human cells, Blood 100(9):3175-3182, 2002), nude mice, and Rag1 and/or Rag2 knockout mice. These mice can optionally be irradiated, or otherwise treated to destroy one or more immune cell type. Thus, in various embodiments, a genetically modified mouse is provided that can include a humanization of at least a portion of an endogenous non-human BTLA locus, and further comprises a modification that compromises, inactivates, or destroys the immune system (or one or more cell types of the immune system) of the non-human animal in whole or in part. In some embodiments, modification is, e.g., selected from the group consisting of a modification that results in NOD mice, SCID mice, NOD/SCID mice, IL-2Rγ knockout mice, NOD/SCID/γc null mice, nude mice, Rag1 and/or Rag2 knockout mice, and a combination thereof. These genetically modified animals are described, e.g., in US20150106961, which is incorporated herein by reference in its entirety. In some embodiments, the mouse can include a replacement of all or part of mature BTLA coding sequence with human mature BTLA coding sequence.

Genetically modified non-human animals that comprise a modification of an endogenous non-human BTLA locus. In some embodiments, the modification can comprise a human nucleic acid sequence encoding at least a portion of a mature BTLA protein (e.g., at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the mature BTLA protein sequence). Although genetically modified cells are also provided that can comprise the modifications described herein (e.g., ES cells, somatic cells), in many embodiments, the genetically modified non-human animals comprise the modification of the endogenous BTLA locus in the germline of the animal.

Genetically modified animals can express a human BTLA and/or a chimeric (e.g., humanized) BTLA from endogenous mouse loci, wherein the endogenous mouse BTLA gene has been replaced with a human BTLA gene and/or a nucleotide sequence that encodes a region of human BTLA sequence or an amino acid sequence that is at least

10%, 20%, 30%, 40%, 50%, 60%, 70&, 80%, 90%, 95%, 96%, 97%, 98%, or 99% identical to the human BTLA sequence. In various embodiments, an endogenous non-human BTLA locus is modified in whole or in part to comprise human nucleic acid sequence encoding at least one protein-coding sequence of a mature BTLA protein.

In some embodiments, the genetically modified mice express the human BTLA and/or chimeric BTLA (e.g., humanized BTLA) from endogenous loci that are under control of mouse promoters and/or mouse regulatory elements. The replacement(s) at the endogenous mouse loci provide non-human animals that express human BTLA or chimeric BTLA (e.g., humanized BTLA) in appropriate cell types and in a manner that does not result in the potential pathologies observed in some other transgenic mice known in the art. The human BTLA or the chimeric BTLA (e.g., humanized BTLA) expressed in animal can maintain one or more functions of the wildtype mouse or human BTLA in the animal. For example, human or non-human HVEM can bind to the expressed BTLA and downregulate immune response, e.g., downregulate immune response by at least 10%, 20%, 30%, 40%, or 50%.Furthermore, in some embodiments, the animal does not express endogenous BTLA. As used herein, the term "endogenous BTLA" refers to BTLA protein that is expressed from an endogenous BTLA nucleotide sequence of the genetically modified non-human animal (e.g., mouse) before the genetic modification.

The genome of the animal can comprise a sequence encoding an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to human BTLA (NP_861445.3) (SEQ ID NO: 27). In some embodiments, the genome comprises a sequence encoding an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31.

The genome of the genetically modified animal can comprise a replacement at an endogenous BTLA gene locus of a sequence encoding a region of endogenous BTLA with a sequence encoding a corresponding region of human BTLA. In some embodiments, the sequence that is replaced is any sequence within the endogenous BTLA gene locus, e.g., exon 1, exon 2, exon 3, exon 4,exon 5, exon 6, 5'-UTR, 3'UTR, the first intron, the second intron, and the third intron, the fourth intron, the fifth intron, the sixth intron etc. In some embodiments, the sequence that is replaced is within the

regulatory region of the endogenous BTLA gene. In some embodiments, the sequence that is replaced is exon2 of an endogenous mouse BTLA gene locus.

The genetically modified animal can have one or more cells expressing a human or chimeric BTLA (e.g., humanized BTLA) having an extracellular region and a cytoplasmic region, wherein the extracellular region comprises a sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, 99% identical to the extracellular region of human BTLA. In some embodiments, the extracellular region of the humanized BTLA has a sequence that has at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 amino acids (e.g., contiguously or non-contiguously) that are identical to human BTLA. Because human BTLA and non-human BTLA (e.g., mouse BTLA) sequences, in many cases, are different, antibodies that bind to human BTLA will not necessarily have the same binding affinity with mouse BTLA or have the same effects to mouse BTLA. Therefore, the genetically modified animal having a human or a humanized extracellular region can be used to better evaluate the effects of anti-human BTLA antibodies in an animal model. In some embodiments, the genome of the genetically modified animal comprises a sequence encoding an amino acid sequence that corresponds to part or the entire sequence of exon 1, exon 2, exon 3, exon 4, and/or exon 5 of human BTLA, part or the entire sequence of extracellular region of human BTLA (with or without signal peptide), or part or the entire sequence of amino acids 34-132 of SEQ ID NO: 27.

In some embodiments, the non-human animal can have, at an endogenous BTLA gene locus, a nucleotide sequence encoding a chimeric human/non-human BTLA polypeptide, wherein a human portion of the chimeric human/non-human BTLA polypeptide comprises a portion of human BTLA extracellular domain, and wherein the animal expresses a functional BTLA on a surface of a cell of the animal. The human portion of the chimeric human/non-human BTLA polypeptide can comprise a portion of exon 1, exon 2, exon 3, exon 4, and/orexon 5 of human BTLA. In some embodiments, the human portion of the chimeric human/non-human BTLA polypeptide can comprise a sequence that is at least 80%, 85%, 90%, 95%, or 99% identical to amino acids 34-132 of SEQ ID NO: 27.

In some embodiments, the non-human portion of the chimeric human/non-human BTLA polypeptide comprises transmembrane and/or cytoplasmic regions of an

endogenous non-human BTLA polypeptide. There may be several advantages that are associated with the transmembrane and/or cytoplasmic regions of an endogenous non-human BTLA polypeptide. For example, once HVEM binds to BTLA, they can properly transmit extracellular signals into the cells and regulate the downstream pathway. A human or humanized transmembrane and/or cytoplasmic regions may not function properly in non-human animal cells. In some embodiments, a few extracellular amino acids that are close to the transmembrane region of BTLA are also derived from endogenous sequence.

Furthermore, the genetically modified animal can be heterozygous with respect to the replacement at the endogenous BTLA locus, or homozygous with respect to the replacement at the endogenous BTLA locus.

In some embodiments, the humanized BTLA locus lacks a human BTLA 5'-UTR. In some embodiment, the humanized BTLA locus comprises a rodent (e.g., mouse) 5'-UTR. In some embodiments, the humanization comprises a human 3'- UTR. In appropriate cases, it may be reasonable to presume that the mouse and human BTLA genes appear to be similarly regulated based on the similarity of their 5'-flanking sequence. As shown in the present disclosure, humanized BTLA mice that comprise a replacement at an endogenous mouse BTLA locus, which retain mouse regulatory elements but comprise a humanization of BTLA encoding sequence, do not exhibit pathologies. Both genetically modified mice that are heterozygous or homozygous for human BTLA are grossly normal.

The present disclosure further relates to a non-human mammal generated through the method mentioned above. In some embodiments, the genome thereof contains human gene(s).

In some embodiments, the non-human mammal is a rodent, and preferably, the non-human mammal is a mouse.

In some embodiments, the non-human mammal expresses a protein encoded by a humanized BTLA gene.

In addition, the present disclosure also relates to a tumor bearing non-human mammal model, characterized in that the non-human mammal model is obtained through

the methods as described herein. In some embodiments, the non-human mammal is a rodent (e.g., a mouse).

The present disclosure further relates to a cell or cell line, or a primary cell culture thereof derived from the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal; the tissue, organ or a culture thereof derived from the non-human mammal or an offspring thereof, or the tumor bearing non-human mammal; and the tumor tissue derived from the non-human mammal or an offspring thereof when it bears a tumor, or the tumor bearing non-human mammal.

The present disclosure also provides non-human mammals produced by any of the methods described herein. In some embodiments, a non-human mammal is provided; and the genetically modified animal contains the DNA encoding human or humanizedBTLA in the genome of the animal.

In some embodiments, the non-human mammal comprises the genetic construct as shown in FIG. 2. In some embodiments, a non-human mammal expressing human or humanized BTLA is provided. In some embodiments, the tissue-specific expression of human or humanizedBTLA protein is provided.

In some embodiments, the expression of human or humanizedBTLA in a genetically modified animal is controllable, as by the addition of a specific inducer or repressor substance.

Non-human mammals can be any non-human animal known in the art and which can be used in the methods as described herein. Preferred non-human mammals are mammals, (e.g., rodents). In some embodiments, the non-human mammal is a mouse.

Genetic, molecular and behavioral analyses for the non-human mammals described above can performed. The present disclosure also relates to the progeny produced by the non-human mammal provided by the present disclosure mated with the same or other genotypes.

The present disclosure also provides a cell line or primary cell culture derived from the non-human mammal or a progeny thereof. A model based on cell culture can be prepared, for example, by the following methods. Cell cultures can be obtained by way of isolation from a non-human mammal, alternatively cell can be obtained from the cell culture established using the same constructs and the standard cell transfection techniques.

The integration of genetic constructs containing DNA sequences encoding human BTLA protein can be detected by a variety of methods.

There are many analytical methods that can be used to detect exogenous DNA expression, including methods at the level of RNA (including the mRNA quantification approaches using reverse transcriptase polymerase chain reaction (RT-PCR) or Southern blotting, and *in situ* hybridization) and methods at the protein level (including histochemistry, immunoblot analysis and *in vitro* binding studies). In addition, the expression level of the gene of interest can be quantified by ELISA techniques well known to those skilled in the art. Many standard analysis methods can be used to complete quantitative measurements. For example, transcription levels can be measured using RT-PCR and hybridization methods including RNase protection, Southern blot analysis, RNA dot analysis (RNAdot) analysis. Immunohistochemical staining, flow cytometry, Western blot analysis can also be used to assess the presence of human BTLA protein.

Vectors

The present disclosure relates to a targeting vector, comprising: a) a DNA fragment homologous to the 5' end of a region to be altered (5' arm), which is selected from the BTLA gene genomic DNAs in the length of 100 to 10,000 nucleotides; b) a desired/donor DNA sequence encoding a donor region; and c) a second DNA fragment homologous to the 3' end of the region to be altered (3' arm), which is selected from the BTLA gene genomic DNAs in the length of 100 to 10,000 nucleotides.

In some embodiments, a) the DNA fragment homologous to the 5' end of a conversion region to be altered (5' arm) is selected from the nucleotide sequences that have at least 90% homology to the NCBI accession number NC_000082.6; c) the DNA fragment homologous to the 3' end of the region to be altered (3' arm) is selected from the nucleotide sequences that have at least 90% homology to the NCBI accession number NC_000082.6.

In some embodiments, a) the DNA fragment homologous to the 5' end of a region to be altered (5' arm) is selected from the nucleotides from the position 45237539 to the position 45239051 of the NCBI accession number NC_000082.6; c) the DNA fragment

homologous to the 3' end of the region to be altered (3' arm) is selected from the nucleotides from the position 45239358 to the position 45240854 of the NCBI accession number NC_000082.6.

In some embodiments, the length of the selected genomic nucleotide sequence in the targeting vector can be about 1.2 kb, about 1.5 kb, or about 1 kb. In some embodiments, the length is about 1513 bp or about 1497 bp.

In some embodiments, the region to be altered is exon 1, exon 2, exon 3, exon 4, exon 5, and/orexon 6 of BTLA gene (e.g., exon 2of BTLA gene).

The targeting vector can further include a selected gene marker.

In some embodiments, the sequence of the 5' arm is shown in SEQ ID NO: 32; and the sequence of the 3' arm is shown in SEQ ID NO: 38.

In some embodiments, the target region is derived from human. For example, the target region in the targeting vector is a part or entirety of the nucleotide sequence of a human BTLA, preferably the nucleotide sequence is shown as a first exon, a second exon, a third exon, a fourth exon, and/or a fifth exonof the DNA sequence of the human BTLA. In some embodiments, the nucleotide sequence of the humanized BTLA encodes the humanized BTLA protein with the NCBI accession number NP_861445.3 (SEQ ID NO: 27).

The disclosure also relates to a cell comprising the targeting vectors as described above.

Moreover, the disclosure also relates to an sgRNA sequence for constructing a humanized animal model, wherein the sgRNA sequence targets the BTLA gene, the sgRNA is unique on the target sequence of the BTLA gene to be altered, and meets the sequence arrangement rule of 5'-NNN (20) -NGG3' or 5'-CCN-N (20)-3'; and in some embodiments, the targeting site of the sgRNA in the mouse BTLA gene is located on the exon 1, exon 2, exon 3,exon 4, exon 5,orexon 6 of the mouse BTLA gene (e.g., exon 2of the mouse BTLA gene).

In some embodiments, an upstream sequence thereof is shown as SEQ ID NO:15, and a downstream sequence thereof is shown as SEQ ID NO: 17, and the sgRNA sequence recognizes a 5' targeting site. In some embodiments, the forward oligonucleotide sequence is obtained by adding TAGG to the 5' end of SEQ ID NO: 15;

and the reverse oligonucleotide sequence is obtained by adding AAAC to the 5' end of SEQ ID NO: 17.

In some embodiments, the disclosure provides an sgRNA sequence for constructing a humanized animal model, wherein an upstream sequence thereof is shown as SEQ ID NO: 19, and a downstream sequence thereof is shown as SEQ ID NO: 21, and the sgRNA sequence recognizes a 3' targeting site. In some embodiments, the forward oligonucleotide sequence is obtained by adding TAGG to the 5' end of SEQ ID NO: 19; and the reverse oligonucleotide sequence is obtained by adding AAAC to the 5' end of SEQ ID NO: 21.

In some embodiments, the disclosure relates to a construct including the sgRNA sequence, and/or a cell including the construct.

In addition, the present disclosure further relates to a non-human mammalian cell, having any one of the foregoing targeting vectors, and one or more *in vitro* transcripts of the sgRNA construct as described herein. In some embodiments, the cell includes Cas9 mRNA or an *in vitro* transcript thereof.

In some embodiments, the genes in the cell are heterozygous. In some embodiments, the genes in the cell are homozygous.

In some embodiments, the non-human mammalian cell is a mouse cell. In some embodiments, the cell is a fertilized egg cell.

Methods of making genetically modified animals

Genetically modified animals can be made by several techniques that are known in the art, including, e.g., nonhomologous end-joining (NHEJ), homologous recombination (HR), zinc finger nucleases (ZFNs), transcription activator-like effector-based nucleases (TALEN), and the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas system. In some embodiments, homologous recombination is used. In some embodiments, CRISPR-Cas9 genome editing is used to generate genetically modified animals. Many of these genome editing techniques are known in the art, and is described, e.g., in Yin et al., "Delivery technologies for genome editing," Nature Reviews Drug Discovery 16.6 (2017): 387-399, which is incorporated by reference in its entirety. Many other methods are also provided and can be used in genome editing, e.g., micro-

injecting a genetically modified nucleus into an enucleated oocyte, and fusing an enucleated oocyte with another genetically modified cell.

Thus, in some embodiments, the disclosure provides replacing in at least one cell of the animal, at an endogenous BTLA gene locus, a sequence encoding a region of an endogenous BTLA with a sequence encoding a corresponding region of human or chimeric BTLA. In some embodiments, the replacement occurs in a germ cell, a somatic cell, a blastocyst, or a fibroblast, etc. The nucleus of a somatic cell or the fibroblast can be inserted into an enucleated oocyte.

FIG. 3C shows a humanization strategy for a mouse BTLA locus. In FIG. 3C, the targeting strategy involves a vector comprising the 5' end homologous arm, human BTLA gene fragment, 3' homologous arm. The process can involve replacing endogenous BTLA sequence with human sequence by homologous recombination. In some embodiments, the cleavage at the upstream and the downstream of the target site (e.g., by zinc finger nucleases, TALEN or CRISPR) can result in DNA double strands break, and the homologous recombination is used to replace endogenous BTLA sequence with human BTLA sequence.

Thus, in some embodiments, the methods for making a genetically modified, humanized animal, can include the step of replacing at an endogenous BTLA locus (or site), a nucleic acid encoding a sequence encoding a region of endogenous BTLA with a sequence encoding a corresponding region of human BTLA.The sequence can include a region (e.g., a part or the entire region) of exon 1, exon 2, exon 3, exon 4, exon 5, and/or exon 6, of a human BTLA gene. In some embodiments, the sequence includes a region of exon 2 of a human BTLA gene (e.g., amino acids 34-132 of SEQ ID NO: 27). In some embodiments, the region is located within the extracellular region of BTLA. In some embodiments, the endogenous BTLA locus is exon2 of mouse BTLA.

In some embodiments, the methods of modifying a BTLA locus of a mouse to express a chimeric human/mouse BTLA peptide can include the steps of replacing at the endogenous mouse BTLA locus a nucleotide sequence encoding a mouse BTLA with a nucleotide sequence encoding a human BTLA, thereby generating a sequence encoding a chimeric human/mouse BTLA.

In some embodiments, the nucleotide sequence encoding the chimeric human/mouse BTLA can include a first nucleotide sequence encoding an extracellular region of mouse BTLA (with or without the mouse signal peptide sequence); a second nucleotide sequence encoding an extracellular region of human BTLA; a third nucleotide sequence encoding a transmembrane and a cytoplasmic region of a mouse BTLA.

In some embodiments, the nucleotide sequences as described herein do not overlap with each other (e.g., the first nucleotide sequence, the second nucleotide sequence, and/or the third nucleotide sequence do not overlap). In some embodiments, the amino acid sequences as described herein do not overlap with each other.

The present disclosure further provides a method for establishing a BTLA gene humanized animal model, involving the following steps:

(a) providing the cell (e.g. a fertilized egg cell) based on the methods described herein;

(b) culturing the cell in a liquid culture medium;

(c) transplanting the cultured cell to the fallopian tube or uterus of the recipient female non-human mammal, allowing the cell to develop in the uterus of the female non-human mammal;

(d) identifying the germline transmission in the offspring genetically modified humanized non-human mammal of the pregnant female in step (c).

In some embodiments, the non-human mammal in the foregoing method is a mouse (e.g., a C57BL/6 mouse).

In some embodiments, the non-human mammal in step (c) is a female with pseudopregnancy (or false pregnancy).

In some embodiments, the fertilized eggs for the methods described above are C57BL/6 fertilized eggs. Other fertilized eggs that can also be used in the methods as described herein include, but are not limited to, FVB/N fertilized eggs, BALB/c fertilized eggs, DBA/1 fertilized eggs and DBA/2 fertilized eggs.

Fertilized eggs can come from any non-human animal, e.g., any non-human animal as described herein. In some embodiments, the fertilized egg cells are derived from rodents. The genetic construct can be introduced into a fertilized egg by microinjection of DNA. For example, by way of culturing a fertilized egg after

microinjection, a cultured fertilized egg can be transferred to a false pregnant non-human animal, which then gives birth of a non-human mammal, so as to generate the non-human mammal mentioned in the method described above.

Methods of using genetically modified animals

Replacement of non-human genes in a non-human animal with homologous or orthologous human genes or human sequences, at the endogenous non-human locus and under control of endogenous promoters and/or regulatory elements, can result in a non-human animal with qualities and characteristics that may be substantially different from a typical knockout-plus-transgene animal. In the typical knockout-plus-transgene animal, an endogenous locus is removed or damaged and a fully human transgene is inserted into the animal's genome and presumably integrates at random into the genome. Typically, the location of the integrated transgene is unknown; expression of the human protein is measured by transcription of the human gene and/or protein assay and/or functional assay. Inclusion in the human transgene of upstream and/or downstream human sequences are apparently presumed to be sufficient to provide suitable support for expression and/or regulation of the transgene.

In some cases, the transgene with human regulatory elements expresses in a manner that is unphysiological or otherwise unsatisfactory, and can be actually detrimental to the animal. The disclosure demonstrates that a replacement with human sequence at an endogenous locus under control of endogenous regulatory elements provides a physiologically appropriate expression pattern and level that results in a useful humanized animal whose physiology with respect to the replaced gene are meaningful and appropriate in the context of the humanized animal's physiology.

Genetically modified animals that express human or humanized BTLA protein, e.g., in a physiologically appropriate manner, provide a variety of uses that include, but are not limited to, developing therapeutics for human diseases and disorders, and assessing the efficacy of these human therapeutics in the animal models.

In various aspects, genetically modified animals are provided that express human or humanized BTLA, which are useful for testing agents that can decrease or block the interaction between BTLA and HVEM or the interaction between BTLA and B7-H4,

testing whether an agent can increase or decrease the immune response, and/or determining whether an agent is an BTLA agonist or antagonist. The genetically modified animals can be, e.g.,an animal model of a human disease, e.g., the disease is induced genetically (a knock-in or knockout). In various embodiments, the genetically modified non-human animals further comprise an impaired immune system, e.g., a non-human animal genetically modified to sustain or maintain a human xenograft, e.g., a human solid tumor or a blood cell tumor (e.g., a lymphocyte tumor, e.g., a B or T cell tumor).

In some embodiments, the genetically modified animals can be used for determining effectiveness of an anti-BTLA antibody for the treatment of cancer. The methods involving administering the anti-BTLA antibody to the animal as described herein, wherein the animal has a tumor; and determining the inhibitory effects of the anti-BTLA antibody to the tumor. The inhibitor effects that can be determined include, e.g., a decrease of tumor size or tumor volume, a decrease of tumor growth, a reduction of the increase rate of tumor volume in a subject (e.g., as compared to the rate of increase in tumor volume in the same subject prior to treatment or in another subject without such treatment), a decrease in the risk of developing a metastasis or the risk of developing one or more additional metastasis, an increase of survival rate, and an increase of life expectancy, etc. The tumor volume in a subject can be determined by various methods, e.g., as determined by direct measurement, MRI or CT.

In some embodiments, the tumor comprises one or more tumor cells that express HVEM (Derré, Laurent, et al. "BTLA mediates inhibition of human tumor-specific CD8+ T cells that can be partially reversed by vaccination." The Journal of clinical investigation 120.1 (2010): 157). In some embodiments, the tumor comprises one or more cancer cells (e.g., human or mouse cancer cells) that are injected into the animal.In some embodiments, the anti-BTLA antibody or anti-HVEM antibody prevents HVEM from binding to BTLA.In some embodiments, the anti-BTLA antibody or anti-HVEM antibody does not prevent HVEMfrom binding to BTLA.

In some embodiments, the genetically modified animals can be used for determining whether an anti-BTLA antibody is anBTLA agonist or antagonist. In some embodiments, the methods as described herein are also designed to determine the effects

of the agent (e.g., anti-BTLA antibodies) on BTLA, e.g., whether the agent can stimulate T cells or inhibit T cells, whether the agent can upregulate the immune response or downregulate immune response.In some embodiments, the genetically modified animals can be used for determining the effective dosage of a therapeutic agent for treating a disease in the subject, e.g., cancer, or autoimmune diseases.

The inhibitory effects on tumors can also be determined by methods known in the art, e.g., measuring the tumor volume in the animal, and/or determining tumor (volume) inhibition rate (TGI_{TV}). The tumor growth inhibition rate can be calculated using the formula TGI_{TV} (%) = (1 – TVt/TVc) x 100, where TVt and TVc are the mean tumor volume (or weight) of treated and control groups.

In some embodiments, the anti-BTLA antibody is designed for treating various cancers. As used herein, the term “cancer” refers to cells having the capacity for autonomous growth, i.e., an abnormal state or condition characterized by rapidly proliferating cell growth. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. The term “tumor” as used herein refers to cancerous cells, e.g., a mass of cancerous cells. Cancers that can be treated or diagnosed using the methods described herein include malignancies of the various organ systems, such as affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. In some embodiments, the agents described herein are designed for treating or diagnosing a carcinoma in a subject. The term “carcinoma” is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. In some embodiments, the cancer is renal carcinoma or melanoma. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term also includes carcinosarcomas, e.g., which include malignant tumors composed of carcinomatous and sarcomatous tissues. An

"adenocarcinoma" refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term "sarcoma" is art recognized and refers to malignant tumors of mesenchymal derivation.

In some embodiments, the anti-BTLA antibody is designed for the treating melanoma, non-small cell lung carcinoma (NSCLC), small cell lung cancer (SCLC), bladder cancer, and/or prostate cancer (e.g., metastatic hormone-refractory prostate cancer). Anti-BTLA antibodies are known in the art, and are described in, e.g., US 8580259, US 8642033, and WO/2016/161415,each of which is incorporated by reference in its entirety.

The present disclosure also relates to the use of the animal model generated through the method as described herein in the development of a product related to an immunization processes of human cells, the manufacturing of a human antibody, or the model system for a research in pharmacology, immunology, microbiology and medicine.

In some embodiments, the disclosure provides the use of the animal model generated through the method as described herein in the production and utilization of an animal experimental disease model of an immunization processes involving human cells, the study on a pathogen, or the development of a new diagnostic strategy and /or a therapeutic strategy.

The disclosure also relates to the use of the animal model generated through the methods as described herein in the screening, verifying, evaluating or studying the BTLA gene function, human BTLA antibodies, drugs for human BTLA targeting sites, the drugs or efficacies for human BTLA targeting sites, the drugs for immune-related diseases and antitumor drugs.

Genetically modified animal model with two or more human or chimeric genes

The present disclosure further relates to methods for generating genetically modified animal model with two or more human or chimeric genes. The animal can comprise a human or chimeric BTLA gene and a sequence encoding an additional human or chimeric protein.

In some embodiments, the additional human or chimeric protein can be programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4

(CTLA-4), Lymphocyte Activating 3 (LAG-3), T-Cell Immunoglobulin And Mucin Domain-Containing Protein 3 (TIM-3), Programmed Cell Death 1 Ligand 1 (PD-L1), TNF Receptor Superfamily Member 9 (4-1BB), CD27, CD28, CD47, T-Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), CD27, Glucocorticoid-Induced TNFR-Related Protein (GITR), or TNF Receptor Superfamily Member 4 (TNFRSF4; or OX40).

The methods of generating genetically modified animal model with two or more human or chimeric genes (e.g., humanized genes) can include the following steps:

(a) using the methods of introducing human BTLA gene or chimeric BTLA gene as described herein to obtain a genetically modified non-human animal;

(b) mating the genetically modified non-human animal with another genetically modified non-human animal, and then screening the progeny to obtain a genetically modified non-human animal with two or more human or chimeric genes.

In some embodiments, in step (b) of the method, the genetically modified animal can be mated with a genetically modified non-human animal with human or chimeric PD-1, CTLA-4, LAG-3, TIM-3, PD-L1, 4-1BB, CD27, CD28, CD47, TIGIT, CD27, GITR, or OX40.

In some embodiments, the BTLA humanization is directly performed on a genetically modified animal having a human or chimeric PD-1, CTLA-4, LAG-3, TIM-3, PD-L1, 4-1BB, CD27, CD28, CD47, TIGIT, CD27, GITR, or OX40 gene.

As these proteins may involve different mechanisms, a combination therapy that targets two or more of these proteins thereof may be a more effective treatment. In fact, many related clinical trials are in progress and have shown a good effect. The genetically modified animal model with two or more human or humanized genes can be used for determining effectiveness of a combination therapy that targets two or more of these proteins, e.g., an anti-BTLA antibody and an additional therapeutic agent for the treatment of cancer. The methods include administering the anti-BTLA antibody and the additional therapeutic agent to the animal, wherein the animal has a tumor; and determining the inhibitory effects of the combined treatment to the tumor.

In some embodiments, the animal further comprises a sequence encoding a human or humanized programmed cell death protein 1 (PD-1). In some embodiments, the

additional therapeutic agent is an anti-PD-1 antibody (e.g., nivolumab, pembrolizumab). In some embodiments, the tumor comprises one or more tumor cells that express HVEM, B7-H4, CD80, CD86, PD-L1 or PD-L2.

In some embodiments, the combination treatment is designed for treating various cancer as described herein, e.g., melanoma, non-small cell lung carcinoma (NSCLC), small cell lung cancer (SCLC), bladder cancer, and/or prostate cancer (e.g., metastatic hormone-refractory prostate cancer).

In some embodiments, the methods described herein can be used to evaluate the combination treatment with some other methods. The methods of treating a cancer that can be used alone or in combination with methods described herein, include, e.g., treating the subject with chemotherapy, e.g., campothecin, doxorubicin, cisplatin, carboplatin, procarbazine, mechlorethamine, cyclophosphamide, adriamycin, ifosfamide, melphalan, chlorambucil, bisulfan, nitrosurea, dactinomycin, daunorubicin, bleomycin, plicomycin, mitomycin, etoposide, verampil, podophyllotoxin, tamoxifen, taxol, transplatinum, 5-flurouracil, vincristin, vinblastin, and/or methotrexate. Alternatively or in addition, the methods can include performing surgery on the subject to remove at least a portion of the cancer, e.g., to remove a portion of or all of a tumor(s), from the patient.

EXAMPLES

The invention is further described in the following examples, which do not limit the scope of the invention described in the claims.

Materials and Methods

The following materials were used in the following examples.

Ambion™ *in vitro* transcription kit was purchased from Ambion.Catalog number is AM1354.

E. coli TOP10 competent cells were purchased from the TiangenBiotech (Beijing) Co.Catalog number is CB104-02.

EcoRI, ScaI, BamHI, BbsI, SacI, StuI, NcoIwere purchased from NEB. Catalog numbers are R3101M, R3122M, R3136M, R0539L, R3156M, R0187M, R3193M.

Kanamycin was purchased from Amresco.Catalog number is 0408.

Cas9 mRNA was obtained from SIGMA.Catalog number is CAS9MRNA-1EA.

AIO kit was obtained from Beijing Biocytogen Co.,Ltd.Catalog number is BCG-DX-004.

UCA kit was obtained from Beijing Biocytogen Co., Ltd. Catalog number is BCG-DX-001.

Reverse Transcription Kit was obtained from TakaRa. Catalog number is 6110A.

C57BL/6 mice were purchased from the China Food and Drugs Research Institute National Rodent Experimental Animal Center.

B-hPD-1 mice were obtained from Beijing Biocytogen Co., Ltd.

Mouse colon cancer cell line MC38 was purchased from Shanghai Enzyme Research Biotechnology Co., Ltd.

Mouse CD3 antibody was obtained from BD.Catalog number is 563123.

mPD-1 antibody was obtained from BIO X CELL. Catalog number is BE0146.

mTcRβPerCP was obtained from Biolegend.Catalog number is 109228.

mPD-1PE was obtained from Biolegend.Catalog number is 109104.

mBTLA PE was obtained from Biolegend. Catalog number is 134804.

hBTLA APC was obtained from Biolegend. Catalog number is 344510.

mCD19 FITC was obtained from Biolegend. Catalog number is 115505.

hPD-1 FITC was obtained from Biolegend.Catalog number is 329904.

EXAMPLE 1:Construction of pT7-BTLA-1 and pT7-BTLA-14

The target sequence determines the targeting specificity of small guide RNA (sgRNA) and the efficiency of Cas9 cleavage at the target gene. Therefore, target sequence selection is important forsgRNA vector construction.

The 5'-terminal targeting sites (sgRNA1 to sgRNA8) and the 3'-terminal targeting sites (sgRNA9 to sgRNA14) were designed and synthesized. The 5'-terminal targeting sites and the 3'-terminal targeting sites are located on exon 2 of mouse BTLA gene, and the targeting site sequence on BTLA of each sgRNA is as follows:

sgRNA-1 targeting sequence: 5'-CAGTGCAACTTACTATTACG-3' (SEQ ID NO: 1)
sgRNA-2 targeting sequence: 5'-CTCGTAATAGTAAGTTGCAC -3' (SEQ ID NO:2)

sgRNA-3 targeting sequence: 5'-GTGACTTGGTGTAAGCACAA-3' (SEQ ID NO:3)
sgRNA-4 targeting sequence: 5'-TCCAAACAGTCTGCCAGGAC-3' (SEQ ID NO:4)
sgRNA-5 targeting sequence: 5'-TTCATAGACCTAATGTGACT-3' (SEQ ID NO:5)
sgRNA-6 targeting sequence: 5'-GGAATTCCAAACAGTCTGCC-3' (SEQ ID NO:6)
sgRNA-7 targeting sequence: 5'-TCCTGTCCTGGCAGACTGTT-3' (SEQ ID NO:7)
sgRNA-8 targeting sequence: 5'-TTTAAATAACTCTCCTGTCC-3' (SEQ ID NO:8)
sgRNA-9 targeting sequence: 5'-TCAGTAACCATCCATGTGAC-3' (SEQ ID NO:9)
sgRNA-10 targeting sequence: 5'-TCACATGGATGGTTACTGAA-3' (SEQ ID NO: 10)
sgRNA-11 targeting sequence: 5'-CCATTATCACTGAGATGTAT-3' (SEQ ID NO: 11)
sgRNA-12 targeting sequence: 5'-CAATACATCTCAGTGATAAT-3' (SEQ ID NO: 12)
sgRNA-13 targeting sequence: 5'-CCAATACATCTCAGTGATAA-3' (SEQ ID NO: 13)
sgRNA-14 targeting sequence: 5'-TGAGATGTATTGGTTTAAAG-3' (SEQ ID NO: 14)

The UCA kit was usedto detect the activitiesofsgRNAs(FIGS. 1A and 1B). The results show that the guide sgRNAs have different activities. Two of them (sgRNA1 and sgRNA14) were selected for follow-up experiments. TAGG was added to the 5' end to obtain a forward oligonucleotide sequence, and its complementary strand was added with AAAC to obtain a reverse oligonucleotide sequence. After annealing, they were respectively digested by restriction enzyme (BbsI) and ligated to pT7- sgRNA plasmid to obtain the expression vectors pT7-BTLA-1and pT7-BTLA-14.

Table 3.sgRNA1and sgRNA14 sequences

sgRNA1 sequences	
SEQ ID NO:15	Upstream:5'-TGCAACTTACTATTACG-3'
SEQ ID NO:16 (adding TAGG to obtain a forward oligonucleotide sequence)	Upstream:5'-TAGGTGCAACTTACTATTACG-3'
SEQ ID NO:17	Downstream:5'-CGTAATAGTAAGTTGCA-3'
SEQ ID NO:18 (complementary strand was added with AAAC to obtain a reverse oligonucleotide sequence)	Downstream:5'-AAACCGTAATAGTAAGTTGCA-3'

sgRNA14	
SEQ ID NO:19	Upstream:5’-AGATGTATTGGTTTAAAG-3’
SEQ ID NO:20 (adding TAGG to obtain a forward oligonucleotide sequence)	Upstream:5’-TAGGAGATGTATTGGTTTAAAG -3’
SEQ ID NO:21	Downstream:5’-CTTTAAACCAATACATCT-3’
SEQ ID NO:22 (complementary strand was added with AAAC to obtain a reverse oligonucleotide sequence)	Downstream:5’-AAACCTTTAAACCAATACATCT-3’

Table 4.The ligation reaction conditions (10μL)

Double stranded fragment	1μL (0.5μM)
pT7-sgRNA vector	1μL (10 ng)
T4 DNA Ligase	1μL (5U)
10×T4 DNA Ligase buffer	1μL
50% PEG4000	1μL
H_2O	Add to 10μL

Reaction conditions:

The ligation reaction was carried outat room temperature for 10 to 30 minutes.The ligation product was then transferred to 30 μL of TOP10 competent cells. The cells were then plated on a petri dish with Kanamycin, and then cultured at 37 °C for at least 12 hours and then two clones were selected and added to LB medium with Kanamycin (5 ml), and then cultured at 37 °C at 250 rpm for at least 12 hours.

Randomly selected clones were sequenced, so as to verify their sequences. The correct expression vectors pT7-B2-6and pT7-B2-10 were selected for subsequent experiments.

Source of pT7-sgRNA plasmid

PT7-sgRNA vector map is shown in FIG. 2. The plasmid backbone was obtainedfrom Takara (Catalog No. 3299). The DNA fragment containing T7 promoter and sgRNA scaffold was synthesized by a plasmid synthesis company, and linked to the backbone vector by restriction enzyme digestion (EcoRI and BamHI) and ligation. The target plasmid was confirmed by the sequencing results.

The DNA fragment containing the T7 promoter and sgRNA scaffold (SEQ ID NO:23):

gaattctaatacgactcactataggggggtcttcgagaagacctgttttagagctagaaatagcaagttaaaataaggct agtccgttatcaacttgaaaaagtggcaccgagtcggtgcttttaaaggatcc

EXAMPLE 2.Construction of vector pClon-4G-BTLA

A partial coding sequence of the mouse BTLA gene (Gene ID:208154) from exon 2(based on the transcript of NCBI accession number NM_001037719.2 → NP_001032808.2 whose mRNA sequence is shown in SEQ ID NO: 24, and the corresponding protein sequence is shown in SEQ ID NO:25) was replaced with a corresponding coding sequence of human homologous BTLA gene (Gene ID:151888) (based on the transcript of NCBI accession number NM_181780.3 →NP_861445.3, whose mRNA sequence was shown in SEQ ID NO: 26, and the corresponding protein sequence is shown in SEQ ID NO:27). The comparison between the mouse BTLA and human BTLAis shown in FIG. 3A, and the finally obtained humanized BTLA gene is shown in FIG. 3B, the humanized mouse BTLA gene DNA sequence (chimeric BTLA gene DNA) is shown in SEQ ID NO: 28.

GATGAAGAG*TGTGATGTACAGCTTTATATAAAGAGACAATCTGAACACTCCAT CTTAGCAGGAGATCCCTTTGAACTAGAATGCCCTGTGAAATACTGTGCTAACAGGC CTCATGTGACTTGGTGCAAGCTCAATGGAACAACATGTGTAAAACTTGAAGATAGA CAAACAAGTTGGAAGGAAGAGAAGAACATTTCATTTTTCATTCTACATTTTGAACCA GTGCTTCCTAATGACAATGGGTCATACCGCTGTTCTGCAAATTTTCAGTCTAATCTC ATTGAAAGCCACTCAACAACTCTTTAT*GTGACAGGTAA

SEQ ID NO: 28 lists only the portion of DNA sequence involved in the modification, wherein the italicized underlined region is the human BTLA gene sequence fragment.

The coding region sequence, mRNA sequence and the encoded protein sequence thereof of the modified humanizedBTLA are respectively shown in SEQ ID NO: 29, SEQ ID NO:30, and SEQ ID NO: 31.

Because human BTLA and mouse BTLA have many isoforms, the methods as described herein can be applied to other isoforms. For example, human BTLA isoform 2 (NCBI accession number NM_001085357.1 (SEQ ID NO: 67) →NP_001078826.1 (SEQ ID NO: 68)) can be used.

A targeting strategy involving a vector comprising the 5' end homologous arm, human BTLA gene fragment, 3' homologous arm as shown in FIG. 3C is also developed. The process is as follows:

(1). Design upstream primers of homologous recombination fragments, and downstream primers matching therewith, as well as other related sequences. Specifically:

5' end homologous arm (SEQ ID NO: 32), nucleotide sequence of the positions from 45237539 to 45239051of the NCBI accession number NC_000082.6 as follows:

Upstream primer (SEQ ID NO: 33):
F:5'-tttaagaaggagatatacatggatatcatacagcaacgacctcgttaagact -3'

Downstream primer (SEQ ID NO: 34):
R:5'-taaagctgtacatcacactcttcatctaaaacaaaaacaaaactgg-3'

(2). Design the primers and related sequences of the desired conversion region. Human DNA fragment (SEQ ID NO: 35) is the nucleotide sequence from positions 112479758 to 112479462of the NCBI accession number NC_000003.12.

The upstream primer (SEQ ID NO: 36) is:
F:5'-gttttagatgaagagtgtgatgtacagctttatataaagagacaa-3'

The downstream primer (SEQ ID NO: 37) is:
R:5'-atcacttacctgtcacataaagagttgttgagtggctttcaatg-3'

(3). Design the upstream primers of the homologous recombination fragment and the downstream primers matching therewith, as well as other related sequences. Specifically:

3' homologous arm (SEQ ID NO: 38), which was the nucleotide sequence from positions 45239358 to 45240854 of the NCBI accession number NC_000082.6:

Upstream primer (SEQ ID NO: 39):
F:5'-cactcaacaactctttatgtgacaggtaagtgatctaccccag-3'
Downstream primer (SEQ ID NO: 40):
R:5'-ttgttagcagccggatctcaggtcgacgctagactatcaattctaccatgttgat-3'

C57BL/6 mouse DNA is used as the template to carry out PCR amplification for the 5'-terminal homologous arm fragment and the 3'-terminal homologous arm fragment. Human DNA is used as the template to carry out PCR amplification for the DNA fragment, and the AIO kit is used to ligate the fragments to the pClon-4G plasmid provided by the kit, so as to obtain the vector pClon-4G-BTLA.

EXAMPLE 3.Verification of vector pClon-4G-BTLA

Three pClon-4G-BTLA clones were randomly selected and identified by three sets of enzymes. Among them,EcoRIshould generate 5779bp+1043bp fragments, SacI should generate 4130bp+2692bp fragments, ScaI should generate 5579bp+1243bp fragments. The results were in line with the expectations (FIG. 4A). The sequences of Plasmids 1, 2, and 3were further verifiedby sequencing. Plasmid 2 was selected for subsequent experiments.

EXAMPLE 4.Microinjection and embryo transfer

The pre-mixed Cas9 mRNA, pClon-4G-BTLA plasmid and *in vitro* transcription products of pT7-BTLA-1 , pT7-BTLA-14 plasmids were injected into the cytoplasm or

nucleus of mouse fertilized eggs (C57BL/6 background) with a microinjection instrument (using*in vitro* transcription kit to carry out the transcription according to the method provided in the product instruction). The embryo microinjection was carried out according to the method described, e.g., in A. Nagy, et al., “Manipulating the Mouse Embryo:A Laboratory Manual (Third Edition),” Cold Spring Harbor Laboratory Press, 2003. The injected fertilized eggs were then transferred to a culture medium for a short time culture, and then was transplanted into the oviduct of the recipient mouse to produce the genetically modified humanized mice (F0 generation). The mice population was further expanded by cross-mating and self-mating to establish stable mouse lines. The humanized mousewas named as B-hBTLA mouse.

EXAMPLE 5. Verification of genetically modified humanized mouse model

1. Genotype determination for F0 generation mice

PCR analysis was performed for mouse tail genomic DNA of F0 generationmice. The primers are for exon 2 of mouse BTLA gene. The primersforPCR-1 were located on the left side of the 5’ homologous arm, the primers for PCR-4 were located on the right side of the 3’ homologous arm; in addition, the primers for PCR-2 and PCR-3 were located on the humanized fragment, which are shown below:

5’ terminus primers:

PCR-1 （SEQ ID NO: 41） ：5’-acttagtggactgtaggagtgctgg-3’

PCR-2 （SEQ ID NO: 42） ：5’-cagcggtatgacccattgtcattagga-3’

3’ terminus primers:

PCR-3 （SEQ ID NO: 43） ：5’-ccatcttagcaggagatccctttga -3’

PCR-4 （SEQ ID NO: 44） ：5’-tagacatgagacaaggttgggcctg -3’

If the recombinant vector has the correct insertion, there should be only one PCR band. The length of the 5’ terminus product should be 1842bp, and the length of the 3’ terminusproductshould be 2428bp.

Table 5.The PCR reaction system (20 μL)

10× buffer	2μL

dNTP (2mM)	2μL
$MgSO_4$ (25mM)	0.8μL
Upstream primer (10μM)	0.6μL
Downstream primer (10μM)	0.6μL
Mouse tailgenomicDNA	200ng
KOD-Plus- (1U/μL)	0.6μL

Table 6. The PCR reaction conditions

Temperature	Time	Cycles
94 °C	5 min	1
94 °C	30 sec	15
67 °C (- 0.7°C /cycle)	30 sec	
68 °C	1 kb/min	
94 °C	30 sec	25
56 °C	30 sec	
68 °C	1 kb/min	
68 °C	10 min	1
4 °C	10 min	1

The verification results for two F0 generation mice are shown in FIG. 5.

2. Genotype determination for F1 generation mice

F1 generation mice were obtained by cross-mating F0 generation mice with C57BL/6 mice. PCR was performed for six F1 generation mice. The results showed that all six F1 generation mice are positive (FIG. 6).

These six mice were furtherexamined by Southern blotting to determine whether they had a random insertion. The genomic DNA was extracted from the mouse tail, and StuI and PstIwere used to digest the genomic DNA. The digestion products were transferred to membrane and hybridized. The probes P1 and P2 were located respectively outside of the 5' homologous arm and in the humanized fragment. The primers for probe synthesis are as follows:

P1-F (SEQ ID NO: 45): 5'- TGATTTGCTTGCTGTTTAAGGTCAT -3'

P1-R (SEQ ID NO: 46): 5'- CTCAGAAAGAGATTTCAAGGGGGTA -3'

P2-F (SEQ ID NO: 47): 5'- GGATGCTCTGATGGGCACACACTTT-3'

P2-R (SEQ ID NO: 48): 5'- TTAGGGAACCAGTTTCTCAGCAGGG-3'

The wild type C56BL/6 mice would havethe11.6kb (P1) and 5.8kb(P2) bands as determined by P1 and P2 probes respectively. The genetically engineered homozygous mice should have the 9.4kb (P1) and 5.8kb (P2) bands ad determined by P1 and P2 probes respectively. The genetically engineered heterozygous mice should have the 11.6kb + 9.4kb (P1) and 5.8kb (P2) bands as determined by P and P2 probes respectively.

The results were shown in FIGS. 7A-7B. Among the six F1 generation mice as determined by P1 probe, F1-4 had no random insertion (FIG. 7A). The results from P2 probe confirmed that F1-4 had no random insertions and F1-4 was ahBTLA heterozygous mouse (FIG. 7B).

It thus shows that this method can be used to generate humanized B-hBTLAmice thathave no random insertion.

3. Protein expression analysis for heterozygous F1 generation mouse

A humanized heterozygous F1 generation mouse was selected for this experiment. One wild type C57BL/6 mouse was used as the control.

7.5 μg of mouse CD3 antibody was injected intraperitoneally to the mice. The spleens were collected 24 hours after the injection, and the spleen samples were grinded.The ground samples were then passed through 70 μm cell mesh, the filtered cell suspensions were centrifuged and the supernatants were discarded; the erythrocyte lysis solution was added for lysis of 5 min, and then PBS solution was added to neutralize the lysis reaction. The solution was centrifuged again and the supernatants were discarded. The cells were washed once with PBS.

FACS: anti-mouse BTLA antibodies (mBTLA PE) and anti-mTCRβ antibodies (TCRβ PerCP), or anti-human BTLA antibodies (hBTLA APC) and anti-mTCRβ antibodies (TCRβ PerCP) were used for staining extracellular proteins. The cells were washed once again with PBS. Flow cytometry was carried out to detect protein expression. Flow cytometry analysis results (FIGS. 8A-8F) show when compared with

the C57BL/6 mice without CD3 antibody stimulation (FIGS. 8A and 8D) or withCD3 antibody stimulation (FIGS. 8B and 8E), the humanized mouse spleen (FIGS. 8C and 8F) has the cells of human BTLA protein expression as detected by anti-human BTLA antibody, while the spleen of the C57BL/6 control mice does not have detectable cells of human BTLA protein expression. The foregoing results indicate that the BTLA genetically modified humanized mouse is able to express human BTLA protein, which can be detected by an anti-human antibody. In contrast, human BTLA protein expression cannot be detected in the C57BL/6 mice.

RT-PCR detection: RNA was extracted from the spleen cells, and cDNA were then obtained by reverse transcription using a reverse transcription kit.

Primers for mBTLA RT-PCR:

mBTLA RT-PCR F1 （SEQ ID NO: 49） ：ACCCCTTGAGGTTAGCCCT, and

mBTLA RT-PCR R1 （SEQ ID NO: 50） ：TTGTAGAACAGCTATACGACCCA

were used to amplify mouse BTLA fragment of 122 bp.

Primers for hBTLA RT-PCR:

hBTLA RT-PCR F1 （SEQ ID NO: 51） ：ATACTGTGCTAACAGGCCTCA， and

hBTLA RT-PCR R1 （SEQ ID NO: 52） ：ACCCATTGTCATTAGGAAGCACT

were used amplify human BTLA fragment of 152 bp.

PCR reaction system was 20 μL, reaction conditions: 95 ℃, 5min; (95 ℃, 30 sec; 60 ℃, 30 sec; 72 ℃, 30 sec, 35 cycles); 72 ℃, 10 min; and then keeping it at 4 ℃. GAPDH was usedas an internal reference.

The results are shown in FIG. 9. The mRNA expression of mouse BTLA was detected in the activated cells of wild-type C57BL/6 mice and F1 generation heterozygous mouse; while the mRNA expression of human BTLA was only detected in the activated cells of the F1 generation heterozygous mouse.

4. Protein expression analysis for B-hBTLA homozygous mice

The B-hBTLAgenetically engineered homozygous mice were obtained by mating the previously obtained heterozygous mice with each other. One homozygous B-hBTLA mouse was selected, and two wild type C57BL/6 mouse were selected as a control. 7.5 μg of mouse CD3 antibody was injected intraperitoneally to the mice, and the spleens of the mice were collected after 24 h. The spleen samples were ground and then filtered through a 70 μm cell filter, the obtained cell suspensions were centrifuged and the resulting supernatants were discarded. The cell samples were added with erythrocyte lysis solution for lysis of 5 min, and then added PBS solution to neutralize the lysis reaction, centrifuged again and the supernatants were discarded, the cells were washed once with PBS. The obtained samples were used in FACS detection and RT-PCR detection.

FACS:The T cells extracellular proteins were simultaneously stained with anti-mouse BTLA antibody (mBTLA PE) and anti-mouse CD19 antibodies (mCD19 FITC) or anti-human BTLA antibody (hBTLA APC) and anti-mouse CD19 antibodies (mCD19 FITC). The cells were then washed with PBS and then detected for protein expression by FACS. Flow cytometry analysis results are shown in FIGS. 10A-10F.The anti-mouse BTLA antibody was able to detect the cells expressing mouse BTLA protein in the spleen samples from the C57BL/6 control mice (FIG. 10B); while the mouse BTLA antibody was unable to detect mouse BTLA protein in the spleen samples from B-hBTLA homozygote (FIG. 10C). Moreover, the human BTLA antibody was able to detect the cells expressing human BTLA protein in the spleen samples from B-hBTLA homozygote (FIG. 10F); while the human BTLA antibody was unable to detect human BTLA protein in the spleen samples from the C57BL/6 control mice (FIG. 10E).

RT-PCR detection: RNA was extracted from the spleen cells of C57BL/6 mice and B-hBTLA homozygotes, and cDNA were then obtained by reverse transcription using a reverse transcription kit. mBTLA RT-PCR F1 （SEQ ID NO: 49） and mBTLA RT-PCR R1 （SEQ ID NO: 50） were used to amplify mouse BTLA fragment of 122 bp.hBTLA RT-PCR F1 （SEQ ID NO: 51） and hBTLA RT-PCR R1 （SEQ ID NO: 52） were used amplify human BTLA fragment of 152 bp.

The results are shown in FIG. 11. The mRNA expression of mouse BTLA was detected in the activated cells of wild-type C57BL/6 mice (+/+); while the mRNA expression of human BTLA was only detected in B-hBTLA homozygotes (H/H).

EXAMPLE 6. BTLA knockout mice

Since the cleavage of Cas9 results in DNA double strands break, and the homologous recombination repair may result in insertion /deletion mutations, it is possible to obtain BTLA knockout mouse when preparing the humanized BTLA mouse. A pair of primers was thus designed. They are located on the left side of the 5' end target site, and to the right side of the 3' end target site, which are shown as follows:

F: 5'-TGAAGAGTGTCCAGTGCAACTTACT-3' (SEQ ID NO: 53)

R: 5'-TGTGGTGGACTGTGGATGTGACAAA-3' (SEQ ID NO: 54)

The PRC reaction systems and conditions are listed in Table 5 and Table 6. Under this condition, the wide type mice should have only one PCR band, and the product length should be about 452bp.The heterozygous mice should have one additional band. The product length should be about 260 bp. The results are shown in FIG. 12. The mice with No. 1-6 are BTLA knockout mice.

EXAMPLE 7. Pharmacological validation of B-hBTLA humanized animal model

B-hBTLA homozygous mice (4-8 weeks) were subcutaneously injected with mouse colon cancer cell MC38 (5×10^5/100 μl PBS), and when the tumor volume grew to about 100 mm^3, the mice were divided to a control group and six treatment groups based on tumor size (n = 5/group). The treatment groups were randomly selected for anti-human BTLA antibodies (AB1, AB2, AB3, AB4, AB5, AB6) treatment (10 mg/kg); the control group was injected with an equal volume of blank solvent. The frequency of administration was twice a week (6 times of administrations in total). The tumor volume was measured twice a week and the body weight of the mice was weighed as well. Euthanasia was performed when the tumor volume of the mouse reached 3000 mm^3.

Overall, the animals in each group were healthy, and the body weights of all the treatment and control group mice increased, and were not significantly different from each other (FIGS. 13 and 14). The tumor in the control group continued growing during

the experimental period; when compared with the control group mice, the tumor volumes in the G3, G4, G5, G6, G7 treatment groups were smaller than the control group G1 (FIG. 15). It thus can be determined that the use of anti-BTLA antibodies (AB2, AB3, AB4, AB5 and AB6) are well tolerated and can inhibit the tumor growth in mice.

Table 7 shows results for this experiment, including the tumor volumes at the day of grouping, 15 days after the grouping, and at the end of the experiment (day 22), the survival rate of the mice, the Tumor Growth Inhibition value (TGI_{TV}), and the statistical differences (P value) in mouse body weights and tumor volume between the treatment and control groups.

Table 7

		Tumor volume (mm^3)			Survival	Non-existence of tumor	TGI_{TV}%	P value	
		Day 0	Day 15	Day 22				Body weight	Tumor Volume
Control	G1	129 ±24	633 ±608	1542 ±1618	5/5	0/5	N/A	N/A	N/A
Treatment	G2(AB1)	129 ±24	820 ±445	1631 ±1093	5/5	0/5	0	0.241	0.921
	G3(AB2)	129 ±22	372 ±247	924 ±641	5/5	0/5	43.8	0.619	0.450
	G4(AB3)	129 ±24	296 ±298	461 ±488	5/5	0/5	76.5	0.724	0.191
	G5(AB4)	129 ±20	480 ±117	624 ±345	5/5	0/5	65.0	0.911	0.250
	G6(AB5)	130 ±23	386 ±317	831 ±881	5/5	0/5	50.4	0.944	0.413
	G7(AB6)	130 ±24	615 ±437	1017 ±839	5/5	0/5	37.2	0.514	0.118

At the end of the experiment (day 22), the body weight of each group increased and there was no significant difference between the groups ($p > 0.05$), indicating that the animals tolerated the six anti-hBTLA antibodies well. With respect to the tumor volume, in the control group (G1), the average tumor volume was 1542±1618 mm^3. The average tumor volumes in the treatment groups were1631±1093 mm^3 (G2), 924±641mm^3 (G3), 461±488mm^3 (G4), 624±345mm^3 (G5), 831±881mm^3 (G6), 1017±839mm^3 (G7).

The tumor volume in the G2 group is not different from the control group (G1), but the tumor volumes in the other treatment groups (G3~G7) were smaller than those in the control group (G1) with TGI_{TV} 43.8%, 76.5%, 76.5%, 65.0%, 50.4%, 37.2% for each treatment group. The results show that anti-human BTLA antibody AB2, AB3, AB4, AB5, AB6have different tumor inhibitory effects in B-hBTLA mice, and AB1 has no tumor inhibitory effects. Under the same condition, the inhibitory effects of AB3(G4) and AB4(G5)are better than AB2, AB5, AB6, and these antibodies have no obvious toxic effects in mice.

The above example has demonstrated that the B-hBTLA mouse model can be used as an *in vivo* animal model for screening, evaluation and study of human BTLA signaling pathway regulators, and test the efficacy of multiple anti-human BTLA antibodies.

EXAMPLE 8. Preparation and identification of mice with double humanized or multiple humanized genes

Mice containing the human BTLA gene (such as the B-hBTLA animal model prepared using the methods as described in the present disclosure) can also be used to prepare an animal model with double-humanized or multi-humanized genes. For example, in Example 4, the fertilized egg cells used in the microinjection and embryo transfer process can be selected from the fertilized egg cells of other genetically modified mice or the fertilized egg cells of B-hBTLA mice, so as to obtain double- or multiple-gene modified mouse models.

In addition, the B-hBTLA animal model homozygote or heterozygote can be mated with other genetically modified homozygous or heterozygous animal models, and the progeny is then screened; according to the Mendelian law, there is a chance to obtain the double-gene or multiple-gene modified heterozygous animal models, and then the obtained heterozygous can be mated with each other to finally obtain the double-gene or multiple-gene modified homozygotes.

In the case of the generating double humanized BTLA/PD-1 mouse, since the mouse BTLA gene and Pd-1 gene are located on different chromosomes, the double

humanized BTLA/PD-1 mouse was obtained by mating the B-hBTLA mouse with B-hPD-1 mouse (mice with humanized PD-1 gene).

PCR analysis was performed on the mouse tail genomic DNA of double humanized BTLA/PD-1 mice using four pairs of primers. The specific sequences and product lengths are shown in Table 8. The reaction system and reaction conditions are shown in Table 9 and Table 10. The results for a number of humanized BTLA/PD-1 mice are shown in FIGS. 16A-16D, wherein FIGS. 16A and 16B show that the mice numbered 3017 to 3032 are BTLA homozygous mice, FIGS. 16C and 16D show that the mice numbered 3017 to 3032 are PD-1 homozygous mice. The results of the two groups indicate that the 16 mice numbered 3017 to 3032 were double-gene homozygotes.

Table 8. Primer sequences

Primer	Sequence	Product length
BTLA WT	F:5'-attgcaatgatacctatggtccttctaagagt-3' (SEQ ID NO: 55)	WT：271bp
	R:5'-ccggaactgattgattttcttccca-3' (SEQ ID NO: 56)	
BTLA MUT	F:5'-gctgaccgtgaacgatacaggg-3' (SEQ ID NO: 57)	Mut：427bp
	R:5'-gaatcggctggttgtttctggaacg-3' (SEQ ID NO: 58)	
PD-1 MUT	F:5'-cttccacatgagcgtggtcagggcc-3' (SEQ ID NO: 59)	Mut：325bp
	R:5'-ccaagggactattttagatgggcag-3' (SEQ ID NO: 60)	
PD-1 WT	F:5'-gaagctacaagctcctaggtaggggg-3' (SEQ ID NO: 61)	WT：345bp
	R:5'-acgggttggctcaaaccattaca-3' (SEQ ID NO: 62)	

Table 9. PCT reaction

2× Master Mix	10μL
Upstream primer（10 μM)	0.5μL
Downstream primer（10μM)	0.5μL
Mouse tail genomic DNA (100-200 ng/20ml)	2 μL
ddH_2O	Add to to 20μL

Table 10. PCR amplification reaction condition

Temperature	Time	Cycles

95 °C	5 min	1
95 °C	30 sec	30
59 °C	30 sec	
72 °C	30 sec	
72 °C	10 min	1
4 °C	10 min	1

The expression of the double humanized BTLA/PD-1 mice was further examined. A double humanized BTLA/PD-1 homozygote (9 weeks old) was selected for the study. Two wild type C57BL/6 mice were selected as control. Mice were injected with 7.5 μg of mouse CD3 antibody intraperitoneally. After 24 hours, the mice were euthanized, and then the spleens of the mice were collected. The spleen samples were ground and the ground samples were filtered through a 70 μm cell mesh. The filtered cell suspensions were centrifuged and the supernatants were discarded; erythrocyte lysis solution was added for lysis for 5 min, and then PBS solution was added to neutralize the lysis reaction. The solution was centrifuged again and the supernatants were discarded, the cells were washed once with PBS. The obtained spleen cell samples were then subject to FACS and RT-PCR analysis.

FACS: The T cells extracellular proteins were simultaneously stained with the following:

(1) anti-mouse BTLA antibody (mBTLA PE)and anti-mouse CD19 antibody (mCD19 FITC) (FIGS. 17A, 17B, 17C);

(2) anti-human BTLA antibody (hBTLA APC) and anti-mouse CD19 antibody (mCD19 FITC) (FIGS. 17D, 17E, 17F);

(3) anti-mouse PD-1 antibody (mPD-1 PE) and mouse T cell surface antibody mTcRβ (FIGS. 18A, 18B, and 18C); or

(4) anti-human PD-1 antibody (hPD-1 FITC) and mouse T cell surface antibody mTcRβ (FIGS. 18D, 18E, and 18F).

The cells were then washed with PBS and then detected for protein expression by FACS. Flow cytometry analysis results are shown in FIGS. 17A-17F and 18A-18F. The anti-human BTLA antibody and the anti-human PD-1 antibody detected the cells

expressing humanized BTLA and humanized PD-1 in humanized BTLA/PD-1 homozygotes. In contrast, the anti-human BTLA antibody and the anti-human PD-1 antibody did not detect cells expressing humanized BTLA and humanized PD-1 in in the spleen samples from the C57BL/6 control mice.

RT-PCR detection: RNA was extracted from the spleen cells of wild-type C57BL/6 mice and humanized BTLA/PD-1 homozygotes. cDNAs were then obtained by reverse transcription using a reverse transcription kit.

mBTLA RT-PCR F1 （SEQ ID NO: 49） and mBTLA RT-PCR R1 （SEQ ID NO: 50） were used to amplify mouse BTLA fragment of 122 bp.

hBTLA RT-PCR F1 （SEQ ID NO: 51） and hBTLA RT-PCR R1 （SEQ ID NO: 52） were used amplify human BTLA fragment of 152 bp.

mPD-1 RT-PCR primer F3: 5'-CCTGGCTCACAGTGTCAGAG-3' （SEQ ID NO: 63） , and mPD-1 RT-PCR primer R3 ：5'-CAGGGCTCTCCTCGATTTTT-3' （SEQ ID NO: 64） were used to amplify mouse PD-1 fragment of 297bp.

hPD-1 RT-PCR primer F3 ：5'-CCCTGCTCGTGGTGACCGAA-3' （SEQ ID NO: 65） ， and hPD-1 RT-PCR primer R3 ：5'-GCAGGCTCTCTTTGATCTGC-3' （SEQ ID NO: 66） were used to amplify human PD-1 fragment of 297bp.

PCR reaction system was 20 μL, reaction conditions: 95 ℃, 5min; (95 ℃, 30 sec; 60 ℃, 30 sec; 72 ℃, 30 sec, 35 cycles); 72 ℃, 10 min; and 4 ℃. GAPDH was used as an internal reference.

The results are shown in FIGS. 19 and 20. The mRNA expression of mouse BTLA and PD-1 can be detected in the activated cells of wild-type C57BL/6 mice; while the mRNA expression of human BTLA and PD-1 can be detected in the activated cells of humanized BTLA/PD-1 homozygous mice.

EXAMPLE 11.Embryonic stem cell based preparation methods

The non-human mammals can also be prepared through other gene editing systems and approaches, which includes, but is not limited to, gene homologous

recombination techniques based on embryonic stem cells (ES), zinc finger nuclease (ZFN) techniques, transcriptional activator-like effector factor nuclease (TALEN) technique, homing endonuclease (megakable base ribozyme), or other molecular biology techniques. In this example, the conventional ES cell gene homologous recombination technique is used as an example to describe how to obtain a BTLA gene humanized mouse by other methods. According to the gene editing strategy of the methods described herein and the humanized mouse BTLA gene map (FIG. 4), a targeting strategy has been developed as shown in FIG. 21. FIG. 21 shows the design of the recombinant vector. In view of the fact that one of the objects is to replace the exon 2 of the mouse BTLA gene in whole or in part with the human BTLA gene fragment, a recombinant vector that contains a 5' homologous arm (3812bp), a 3' homologous arm (4169bp) and a humanized gene fragment (297bp) is also designed. The vector can also contain a resistance gene for positive clone screening, such as neomycin phosphotransferase coding sequence Neo. On both sides of the resistance gene, two site-specific recombination systems in the same orientation, such as Frt or LoxP, can be added. Furthermore, a coding gene with a negative screening marker, such as the diphtheria toxin A subunit coding gene (DTA), can be constructed downstream of the recombinant vector 3' homologous arm. Vector construction can be carried out using methods known in the art, such as enzyme digestion and so on. The recombinant vector with correct sequence can be next transfected into mouse embryonic stem cells, such as C57BL/6 mouse embryonic stem cells, and then the recombinant vector can be screened by positive clone screening gene. The cells transfected with the recombinant vector are next screened by using the positive clone marker gene, and Southern Blot technique can be used for DNA recombination identification. For the selected correct positive clones, the positive clonal cells (black mice) are injected into the isolated blastocysts (white mice) by microinjection according to the method described in the book A. Nagy, et al., "Manipulating the Mouse Embryo: A Laboratory Manual (Third Edition)," Cold Spring Harbor Laboratory Press, 2003. The resulting chimeric blastocysts formed following the injection are transferred to the culture medium for a short time culture and then transplanted into the fallopian tubes of the recipient mice (white mice) to produce F0 generation chimeric mice (black and white). The F0 generation chimeric mice with correct gene recombination are then selected by

extracting the mouse tail genome and detecting by PCR for subsequent breeding and identification. The F1 generation mice are obtained by mating the F0 generation chimeric mice with wild type mice. Stable gene recombination positive F1 heterozygous mice are selected by extracting rat tail genome and PCR detection. Next, the F1 heterozygous mice are mated to each other to obtain genetically recombinant positive F2 generation homozygous mice. In addition, the F1 heterozygous mice can also be mated with Flp or Cre mice to remove the positive clone screening marker gene (neo, etc.), and then the BTLA gene humanized homozygous mice can be obtained by mating these mice with each other. The methods of genotyping and phenotypic detection of the obtained F1 heterozygous mice or F2 homozygous mice are similar to those used in Example 5 described above.

OTHER EMBODIMENTS

It is to be understood that while the invention has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the invention, which is defined by the scope of the appended claims.Other aspects, advantages, and modifications are within the scope of the following claims.

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Leu Leu Leu Ala Cys Val Cys Leu Leu Cys Phe Leu Lys Arg Ile Gln
195 200 205

Gly Lys Glu Lys Lys Pro Ser Asp Leu Ala Gly Arg Asp Thr Asn Leu
210 215 220

Val Asp Ile Pro Ala Ser Ser Arg Thr Asn His Gln Ala Leu Pro Ser
225 230 235 240

Gly Thr Gly Ile Tyr Asp Asn Asp Pro Trp Ser Ser Met Gln Asp Glu
245 250 255

Ser Glu Leu Thr Ile Ser Leu Gln Ser Glu Arg Asn Asn Gln Gly Ile
260 265 270

Val Tyr Ala Ser Leu Asn His Cys Val Ile Gly Arg Asn Pro Arg Gln

275 280 285

Glu Asn Asn Met Gln Glu Ala Pro Thr Glu Tyr Ala Ser Ile Cys Val
290 295 300

Arg Ser
305

<210> 26
<211> 3216
<212> DNA/RNA
<213> human

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tccatcactg atatgtgcag gaaatgaaga cattgcctgc catgcttgga actgggaaat 240

tattttgggt cttcttctta atcccatatc tggacatctg gaacatccat gggaaagaat 300

catgtgatgt acagctttat ataaagagac aatctgaaca ctccatctta gcaggagatc 360

cctttgaact agaatgccct gtgaaatact gtgctaacag gcctcatgtg acttggtgca 420

agctcaatgg aacaacatgt gtaaaacttg aagatagaca aacaagttgg aaggaagaga 480

agaacatttc atttttcatt ctacattttg aaccagtgct tcctaatgac aatgggtcat 540

accgctgttc tgcaaatttt cagtctaatc tcattgaaag ccactcaaca actctttatg 600

tgacagatgt aaaaagtgcc tcagaacgac cctccaagga cgaaatggca agcagaccct 660

ggctcctgta tagtttactt cctttggggg gattgcctct actcatcact acctgtttct 720

gcctgttctg ctgcctgaga aggcaccaag gaaagcaaaa tgaactctct gacacagcag 780

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gtttcaggat gcaggaaggg tctgaagttt attctaatcc atgcctggaa gaaaacaaac 960

caggcattgt ttatgcttcc ctgaaccatt ctgtcattgg accgaactca agactggcaa 1020

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<210> 27
<211> 289
<212> PRT
<213> human

<400> 27

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20 25 30

Ser Cys Asp Val Gln Leu Tyr Ile Lys Arg Gln Ser Glu His Ser Ile
35 40 45

Leu Ala Gly Asp Pro Phe Glu Leu Glu Cys Pro Val Lys Tyr Cys Ala
50 55 60

Asn Arg Pro His Val Thr Trp Cys Lys Leu Asn Gly Thr Thr Cys Val
65 70 75 80

Lys Leu Glu Asp Arg Gln Thr Ser Trp Lys Glu Glu Lys Asn Ile Ser
85 90 95

Phe Phe Ile Leu His Phe Glu Pro Val Leu Pro Asn Asp Asn Gly Ser
100 105 110

Tyr Arg Cys Ser Ala Asn Phe Gln Ser Asn Leu Ile Glu Ser His Ser
115 120 125

Thr Thr Leu Tyr Val Thr Asp Val Lys Ser Ala Ser Glu Arg Pro Ser
130 135 140

Lys Asp Glu Met Ala Ser Arg Pro Trp Leu Leu Tyr Ser Leu Leu Pro
145 150 155 160

Leu Gly Gly Leu Pro Leu Leu Ile Thr Thr Cys Phe Cys Leu Phe Cys
165 170 175

Cys Leu Arg Arg His Gln Gly Lys Gln Asn Glu Leu Ser Asp Thr Ala
180 185 190

Gly Arg Glu Ile Asn Leu Val Asp Ala His Leu Lys Ser Glu Gln Thr
195 200 205

Glu Ala Ser Thr Arg Gln Asn Ser Gln Val Leu Leu Ser Glu Thr Gly
210 215 220

Ile Tyr Asp Asn Asp Pro Asp Leu Cys Phe Arg Met Gln Glu Gly Ser
225 230 235 240

Glu Val Tyr Ser Asn Pro Cys Leu Glu Glu Asn Lys Pro Gly Ile Val
245 250 255

Tyr Ala Ser Leu Asn His Ser Val Ile Gly Pro Asn Ser Arg Leu Ala
260 265 270

Arg Asn Val Lys Glu Ala Pro Thr Glu Tyr Ala Ser Ile Cys Val Arg
275 280 285

Ser

<210> 28
<211> 317
<212> DNA
<213> Artificial Sequence

<400> 28
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tggtgcaagc tcaatggaac aacatgtgta aaacttgaag atagacaaac aagttggaag 180

gaagagaaga acatttcatt tttcattcta cattttgaac cagtgcttcc taatgacaat 240

gggtcatacc gctgttctgc aaattttcag tctaatctca ttgaaagcca ctcaacaact 300

ctttatgtga caggtaa 317

<210> 29
<211> 912

<212> DNA/RNA
<213> Artificial Sequence

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gatgtacagc tttatataaa gagacaatct gaacactcca tcttagcagg agatcccttt 180

gaactagaat gccctgtgaa atactgtgct aacaggcctc atgtgacttg gtgcaagctc 240

aatggaacaa catgtgtaaa acttgaagat agacaaacaa gttggaagga agagaagaac 300

atttcatttt tcattctaca ttttgaacca gtgcttccta atgacaatgg gtcataccgc 360

tgttctgcaa attttcagtc taatctcatt gaaagccact caacaactct ttatgtgaca 420

gaaaggactc aaaactcttc agaacaccca ctaataacag tatctgacat cccagatgcc 480

accaatgcct caggaccatc caccatggaa gagaggccag gcaggacttg gctgctttac 540

accttgcttc ctttgggggc attgcttctg ctccttgcct gtgtctgcct gctctgcttt 600

ctgaaaagga tccaagggaa agaaaagaag ccttctgact tggcaggaag ggacactaac 660

ctggttgata ttccagccag ttccaggaca aatcaccaag cactgccatc aggaactgga 720

atttatgata atgatccctg gtctagcatg caggatgaat ctgaattgac aattagcttg 780

caatcagaga gaaacaacca gggcattgtt tatgcttctt tgaaccattg tgttattgga 840

aggaatccaa gacaggaaaa caacatgcag gaggcaccca cagaatatgc atccatttgt 900

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<210> 30
<211> 3226
<212> DNA/RNA
<213> Artificial Sequence

<400> 30
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gaatgatgaa gagtgtgatg tacagcttta tataaagaga caatctgaac actccatctt 180

agcaggagat ccctttgaac tagaatgccc tgtgaaatac tgtgctaaca ggcctcatgt 240

gacttggtgc aagctcaatg gaacaacatg tgtaaaactt gaagatagac aaacaagttg 300

gaaggaagag aagaacattt catttttcat tctacatttt gaaccagtgc ttcctaatga 360

caatgggtca taccgctgtt ctgcaaattt tcagtctaat ctcattgaaa gccactcaac 420

aactctttat gtgacagaaa ggactcaaaa ctcttcagaa cacccactaa taacagtatc 480

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ctgcctgctc tgctttctga aaaggatcca agggaaagaa aagaagcctt ctgacttggc 660

aggaagggac actaacctgg ttgatattcc agccagttcc aggacaaatc accaagcact 720

gccatcagga actggaattt atgataatga tccctggtct agcatgcagg atgaatctga 780

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atatgcatcc atttgtgtga gaagttaaac ctgccactga gccaggcagc ctacactgca 960

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cattcattaa tgcctgtttc ttgattctga ggaagtgaat cagaaaaaat catatgtaat 1920

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cctcaaataa atttgttcca gcattatttt catggttacc taactc 3226

<210> 31
<211> 303
<212> PRT
<213> Artificial Sequence

<400> 31

Met Lys Thr Val Pro Ala Met Leu Gly Thr Pro Arg Leu Phe Arg Glu
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Phe Phe Ile Leu His Leu Gly Leu Trp Ser Ile Leu Cys Glu Lys Ala
20 25 30

Thr Lys Arg Asn Asp Glu Glu Cys Asp Val Gln Leu Tyr Ile Lys Arg
35 40 45

Gln Ser Glu His Ser Ile Leu Ala Gly Asp Pro Phe Glu Leu Glu Cys
50 55 60

Pro Val Lys Tyr Cys Ala Asn Arg Pro His Val Thr Trp Cys Lys Leu
65 70 75 80

Asn Gly Thr Thr Cys Val Lys Leu Glu Asp Arg Gln Thr Ser Trp Lys
85 90 95

Glu Glu Lys Asn Ile Ser Phe Phe Ile Leu His Phe Glu Pro Val Leu

100 105 110

Pro Asn Asp Asn Gly Ser Tyr Arg Cys Ser Ala Asn Phe Gln Ser Asn
115 120 125

Leu Ile Glu Ser His Ser Thr Thr Leu Tyr Val Thr Glu Arg Thr Gln
130 135 140

Asn Ser Ser Glu His Pro Leu Ile Thr Val Ser Asp Ile Pro Asp Ala
145 150 155 160

Thr Asn Ala Ser Gly Pro Ser Thr Met Glu Glu Arg Pro Gly Arg Thr
165 170 175

Trp Leu Leu Tyr Thr Leu Leu Pro Leu Gly Ala Leu Leu Leu Leu Leu
180 185 190

Ala Cys Val Cys Leu Leu Cys Phe Leu Lys Arg Ile Gln Gly Lys Glu
195 200 205

Lys Lys Pro Ser Asp Leu Ala Gly Arg Asp Thr Asn Leu Val Asp Ile
210 215 220

Pro Ala Ser Ser Arg Thr Asn His Gln Ala Leu Pro Ser Gly Thr Gly
225 230 235 240

Ile Tyr Asp Asn Asp Pro Trp Ser Ser Met Gln Asp Glu Ser Glu Leu
245 250 255

Thr Ile Ser Leu Gln Ser Glu Arg Asn Asn Gln Gly Ile Val Tyr Ala
260 265 270

Ser Leu Asn His Cys Val Ile Gly Arg Asn Pro Arg Gln Glu Asn Asn
275 280 285

Met Gln Glu Ala Pro Thr Glu Tyr Ala Ser Ile Cys Val Arg Ser
290 295 300

<210> 32
<211> 1513
<212> DNA
<213> Artificial Sequence

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ttttgggaaa gcatgaaatt gtttgtttgt ttgtttgttt gtttgaggca gtgcttctct 180

ttgtatccct cgctgtccta gaacttactc tgtagatcag gctggccttg aactcagaga 240

tctgcctgcc tctgcttcca gagatcttgg attaaaggca ttcaccacca tcacccagta 300

aaataatttt ttaaaaatat ttttattagg tattttcctc atttacattt ccaatgctat 360

cccaaaagtc ccccataccc tccaccccca ctcccctacc cactcactcc cactttttgg 420

ccctggtgtt cccctgtact ggggcatata aagtttgtgt gtccaatggg cctctctttc 480

caatgatggc tgactaggcc atcttttgat acatatgcag ctagagtcaa gagctcaggg 540

gtactggtta gatcataatg ttgttccatc tatagggttg cagatccctt tagctccttg 600

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catccacttc tgtgtttgct aggccccagc atagtctcac aagagtcagc tctatctggg 720

tcctttcagc aaaatcttgc tagtgtgtac aatggtgtca gtgtttggaa gctgattatg 780

ggatggatcc ctggagatgg cagtctctag atggtccatc cttttgtcac agctccaaac 840

attgtctctg taactccttc catgggtgtt ttgttcccaa ttctaagaag gggcaaagtg 900

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ttaaggcaag catttattac gtgacagttg tgtaccacac tggaatactg aaggtaaagt 1380

atagttaaga aaattgaagc aaattataga attaaacatg agaaaaactt tagtgaaaat 1440

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tttagatgaa gag 1513

<210> 33
<211> 52
<212> DNA
<213> Artificial Sequence

<400> 33
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<210> 34
<211> 46
<212> DNA
<213> Artificial Sequence

<400> 34
taaagctgta catcacactc ttcatctaaa acaaaaacaa aactgg 46

<210> 35
<211> 297
<212> DNA
<213> human

<400> 35
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ctcaatggaa caacatgtgt aaaacttgaa gatagacaaa caagttggaa ggaagagaag 180

aacatttcat ttttcattct acattttgaa ccagtgcttc ctaatgacaa tgggtcatac 240

cgctgttctg caaattttca gtctaatctc attgaaagcc actcaacaac tctttat 297

<210> 36
<211> 45
<212> DNA
<213> Artificial Sequence

<400> 36
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<210> 37
<211> 44
<212> DNA
<213> Artificial Sequence

<400> 37
atcacttacc tgtcacataa agagttgttg agtggctttc aatg 44

<210> 38
<211> 1497
<212> DNA
<213> Artificial Sequence

<400> 38
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acatccacag tccaccacat ccccccagat cagaacagtc ataagcacat aaaatagggg 180

agaagactgt gaagccaaga acagtagaag aaaaataatg gaaatcacta gtctcttggg 240

agaaaggttt acttgtataa ccctgtctgt cctggaactc acgttgtaga ccaggctagc 300

cttgaattca cagagatctg tctaccgctg cctcctgagt gcttggatta aaagcacgta 360

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ttgacaagtt caaaaataga tcacaagtat tattttcttt tttgtattca tttttttctg 480

ttgttgactt ttattgatta gagtttatag aagttatgtt tggatgctct gatgggcaca 540

cactttttaa agtactgatt catactttgc tcacaggacg aagaacatgc aaataagtgc 600

atgttttgat tccttgttga gttttaggga ataggcagag tttccacgtc ctcccattcc 660

ctgagttttt ccattggttc taggagcaaa gacaagacag gcaggtagta agctgggaac 720

actcaaggtg aacaaaacct agacaagtaa aaatgaaaag gcaggaataa tagagggtaa 780

caatcagctt atggacatct atctccaata ctatactccc tgctgagaaa ctggttccct 840

aaagaatgga aacttccatt catcactgag ctggaagctc caactagaaa aatttgacaa 900

gactaagaaa taaaaggtac tgaaaatgga atgggagtaa aagaatctct tttcatgcat 960

gaaatgacct tatatttaga agaaaatgta taaatgtcca taaaactact aacattaata 1020

tatgagttca attttaggaa actgtattaa cacataagaa ctaatgggct ctacatacta 1080

gcaatggata atctgaaaag gttgttaagc aaacaattcc acttataaca gatcaaaaag 1140

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gaaattaagg aagatataaa aatatcatat tgtcatcagt ttcaaagact taactgttat 1260

ggagatgaca gtccccaaaa tccaacatca tttttatcaa ctcatacttc tgaaaccttt 1320

taaaagagat gtaagagcta attctagaat tcacatcata ttggaataga ccagaatagt 1380

caaaataaac ttgataaaga tgggaattgg aagagctctc tatttcaaaa cttattatta 1440

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<210> 39
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Ser

WHAT IS CLAIMED IS:

1. A genetically-modified, non-human animal whose genome comprises at least one chromosome comprising a sequence encoding a human or chimeric B and T Lymphocyte Associated (BTLA).

2. The animal of claim 1, wherein the sequence encoding the human or chimeric BTLA is operably linked to an endogenous regulatory element at the endogenous BTLA gene locus in the at least one chromosome.

3. The animal of claim 1, wherein the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to human BTLA (NP_861445.3 (SEQ ID NO: 27)).

4. The animal of claim 1, wherein the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that is at least 70%, 75%, 80%, 85%, 90%, 95%, 99%, or 100% identical to SEQ ID NO: 31.

5. The animal of claim 1, wherein the sequence encoding a human or chimeric BTLA comprises a sequence encoding an amino acid sequence that corresponds to amino acids 34-132 of SEQ ID NO: 27.

6. The animal of any one of claims 1-5, wherein the animal is a mammal, e.g., a monkey, a rodent or a mouse.

7. The animal of any one of claims 1-5, wherein the animal is a C57BL/6 mouse.

8. The animal of any one of claims 1-7, wherein the animal does not express endogenous BTLA.

9. The animal of claim 1, wherein the animal has one or more cells expressing human or chimeric BTLA.

10. The animal of claim 1, wherein the animal has one or more cells expressing human or chimeric BTLA, and human HVEM can bind to the expressed human or chimeric BTLA.

11. The animal of claim 1, wherein the animal has one or more cells expressing human or chimeric BTLA, and endogenous HVEM can bind to the expressed human or chimeric BTLA.

12. A genetically-modified, non-human animal, wherein the genome of the animal comprises a replacement, at an endogenous BTLA gene locus, of a sequence encoding a region of endogenous BTLA with a sequence encoding a corresponding region of human BTLA.

13. The animal of claim 12, wherein the sequence encoding the corresponding region of human BTLA is operably linked to an endogenous regulatory element at the endogenous BTLA locus, and one or more cells of the animal expresses a chimeric BTLA.

14. The animal of claim 12, wherein the animal does not express endogenous BTLA.

15. The animal of claim 12, wherein the region of endogenous BTLA is the extracellular region of BTLA.

16. The animal of claim 12, wherein the animal has one or more cells expressing a chimeric BTLA having an extracellular region, a transmembrane region, and a cytoplasmic region, wherein the extracellular region comprises a sequence that is at least 50%, 60%, 70%, 80%, 90%, 95%, or 99% identical to the extracellular region of human BTLA.

17. The animal of claim 16, wherein the extracellular region of the chimeric BTLA has a sequence that has at least 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100 contiguous amino acids that are identical to a contiguous sequence present in the extracellular region of human BTLA.

18. The animal of claim 12, wherein the animal is a mouse, and the sequence encoding the region of endogenous BTLA is exon 1, exon 2, exon 3, exon 4, exon 5, and/orexon 6 of the endogenous mouse BTLA gene.

19. The animal of claim 12, wherein the animal is heterozygous with respect to the replacement at the endogenous BTLA gene locus.

20. The animal of claim 12, wherein the animal is homozygous with respect to the replacement at the endogenous BTLA gene locus.

21. A method for making a genetically-modified, non-human animal, comprising:
    replacing in at least one cell of the animal, at an endogenous BTLA gene locus, a sequence encoding a region of an endogenous BTLA with a sequence encoding a corresponding region of human BTLA.

22. The method of claim 21, wherein the sequence encoding the corresponding region of human BTLA comprises exon 1, exon 2, exon 3, exon 4, and/or exon 5 of a human BTLA gene.

23. The method of claim 21, wherein the sequence encoding the corresponding region of BTLA comprises a part of exon 2 of a human BTLA gene.

24. The method of claim 21, wherein the sequence encoding the corresponding region of human BTLA encodes amino acids 34-132 of SEQ ID NO: 27.

25. The method of claim 21, wherein the region is located within the extracellular region of BTLA.

26. The method of claim 21, wherein the animal is a mouse, and the sequence encoding the region of the endogenous BTLA locus is exon2 of the mouse BTLA gene.

27. A non-human animal comprising at least one cell comprising a nucleotide sequence encoding a chimeric BTLA polypeptide, wherein the chimeric BTLA polypeptide comprises at least 50 contiguous amino acid residues that are identical to the corresponding contiguous amino acid sequence of a human BTLA, wherein the animal expresses the chimeric BTLA.

28. The animal of claim 27, wherein the chimeric BTLA polypeptide has at least 50 contiguous amino acid residues that are identical to the corresponding contiguous amino acid sequence of a human BTLA extracellular region.

29. The animal of claim 27, wherein the chimeric BTLA polypeptide comprises a sequence that is at least 90%, 95%, or 99% identical to amino acids 34-132 of SEQ ID NO: 27.

30. The animal of claim 27, wherein the nucleotide sequence is operably linked to an endogenous BTLA regulatory element of the animal.

31. The animal of claim 27, wherein the chimeric BTLA polypeptide comprises an endogenous BTLAtransmembrane region and/or an endogenous BTLA cytoplasmic region.

32. The animal of claim 27, wherein the nucleotide sequence is integrated to an endogenous BTLA gene locus of the animal.

33. The animal of claim 27, wherein the chimeric BTLA has at least one mouse BTLA activity and/or at least one human BTLA activity.

34. A method of making a genetically-modified mouse cell that expresses a chimeric BTLA, the method comprising:
replacing, at an endogenous mouse BTLA gene locus, a nucleotide sequence encoding a region of mouse BTLA with a nucleotide sequence encoding a corresponding region of human BTLA, thereby generating a genetically-modified mouse cell that includes a nucleotide sequence that encodes the chimeric BTLA, wherein the mouse cell expresses the chimeric BTLA.

35. The method of claim 34, wherein the chimeric BTLA comprises
an extracellular region of mouse BTLA comprising a mouse signal peptide sequence;
an extracellular region of human BTLA;
a transmembrane and/or a cytoplasmic region of a mouse BTLA.

36. The method of claim 35, wherein the nucleotide sequence encoding the chimeric BTLA is operably linked to an endogenous BTLA regulatory region, e.g., promoter.

37. The animal of any one of claims 1-20 and 27-33, wherein the animal further comprises a sequence encoding an additional human or chimeric protein.

38. The animal of claim 37, wherein the additional human or chimeric protein is programmed cell death protein 1 (PD-1), cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), Lymphocyte Activating 3 (LAG-3), T-Cell Immunoglobulin And Mucin Domain-Containing Protein 3 (TIM-3), Programmed Cell Death 1 Ligand 1 (PD-L1), TNF Receptor Superfamily Member 9 (4-1BB), CD27, CD28, CD47, T-Cell Immunoreceptor With Ig And ITIM Domains (TIGIT), CD27, Glucocorticoid-Induced TNFR-Related Protein (GITR), or TNF Receptor Superfamily Member 4 (OX40).

39. The method of any one of claims 21-26 and 34-36, wherein the animal or mouse further comprises a sequence encoding an additional human or chimeric protein.

40. The method of claim 39, wherein the additional human or chimeric protein is programmed cell death protein 1 (PD-1), CTLA-4, LAG-3, TIM-3, PD-L1, 4-1BB, CD27, CD28, CD47, TIGIT, CD27, GITR, or OX40.

41. A method of determining effectiveness of an anti-BTLA antibody for the treatment of cancer, comprising:
administering the anti-BTLA antibody to the animal of any one of claims 1-20 and 27-33, wherein the animal has a tumor; and
determining the inhibitory effects of the anti-BTLA antibody to the tumor.

42. The method of claim 41, wherein the tumor comprises one or more tumor cells that express HVEM or B7-H4.

43. The method of claim 41, wherein the tumor comprises one or more cancer cells that are injected into the animal.

44. The method of claim 41, wherein determining the inhibitory effects of the anti-BTLA antibody to the tumor involves measuring the tumor volume in the animal.

45. The method of claim 41, wherein the tumor cells are melanoma cells, non-small cell lung carcinoma (NSCLC) cells, small cell lung cancer (SCLC) cells, bladder cancer cells, and/or prostate cancer cells (e.g., metastatic hormone-refractory prostate cancer).

46. A method of determining effectiveness of an anti-BTLA antibody and an additional therapeutic agent for the treatment of a tumor, comprising
administering the anti-BTLA antibody and the additional therapeutic agent to the animal of any one of claims 1-20 and 27-33, wherein the animal has a tumor; and

determining the inhibitory effects on the tumor.

47. The method of claim 46, wherein the animal further comprises a sequence encoding a human or chimeric programmed cell death protein 1 (PD-1).

48. The method of claim 46, wherein the additional therapeutic agent is an anti-PD-1 antibody.

49. The method of claim 46, wherein the tumor comprises one or more tumor cells that express HVEM or B7-H4.

50. The method of claim 46, wherein the tumor comprises one or more tumor cells that express PD-L1 or PD-L2.

51. The method of claim 46, wherein the tumor is caused by injection of one or more cancercellsinto the animal.

52. The method of claim 46, wherein determining the inhibitory effects of the treatment involves measuring the tumor volume in the animal.

53. The method of claim 46, wherein the tumor comprises melanoma cells, non-small cell lung carcinoma (NSCLC) cells, small cell lung cancer (SCLC) cells, bladder cancer cells, and/or prostate cancer cells (e.g., metastatic hormone-refractory prostate cancer cells).

54. A protein comprising an amino acid sequence, wherein the amino acid sequence is one of the following:
    (a) an amino acid sequence set forth in SEQ ID NO: 31;
    (b) an amino acid sequence that is at least 90% identical to SEQ ID NO: 31;
    (c) an amino acid sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 31;

(d) an amino acid sequence that is different from the amino acid sequence set forth in SEQ ID NO: 31 by no more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 amino acid; and
(e) an amino acid sequence that comprises a substitution, a deletion and /or insertion of one, two, three, four, five or more amino acids to the amino acid sequence set forth in SEQ ID NO: 31.

55. A nucleic acid comprising a nucleotide sequence, wherein the nucleotide sequence is one of the following:
(a) a sequence that encodes the protein of claim 54;
(b) SEQ ID NO: 29;
(c) SEQ ID NO: 30;
(d) a sequence that is at least 90% identical to SEQ ID NO: 29 or SEQ ID NO: 30;
(e) a sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 29; and
(f) a sequence that is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 30;

56. A cell comprising the protein of claim 54 and/or the nucleic acid of claim 55.

57. An animal comprising the protein of claim 54 and/or the nucleic acid of claim 55.

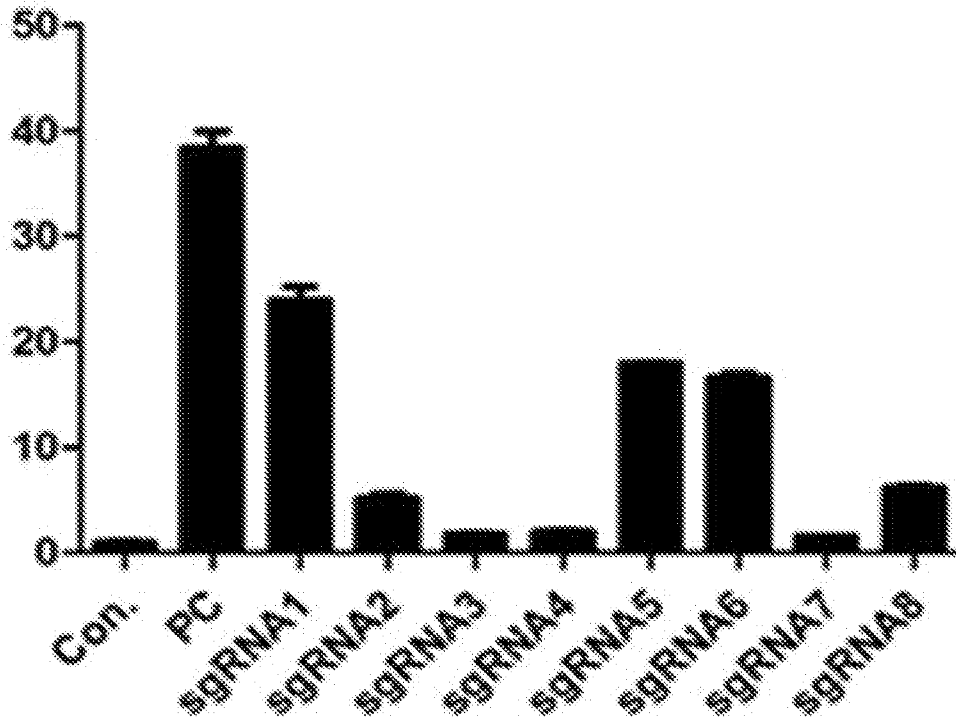


FIG. 1A

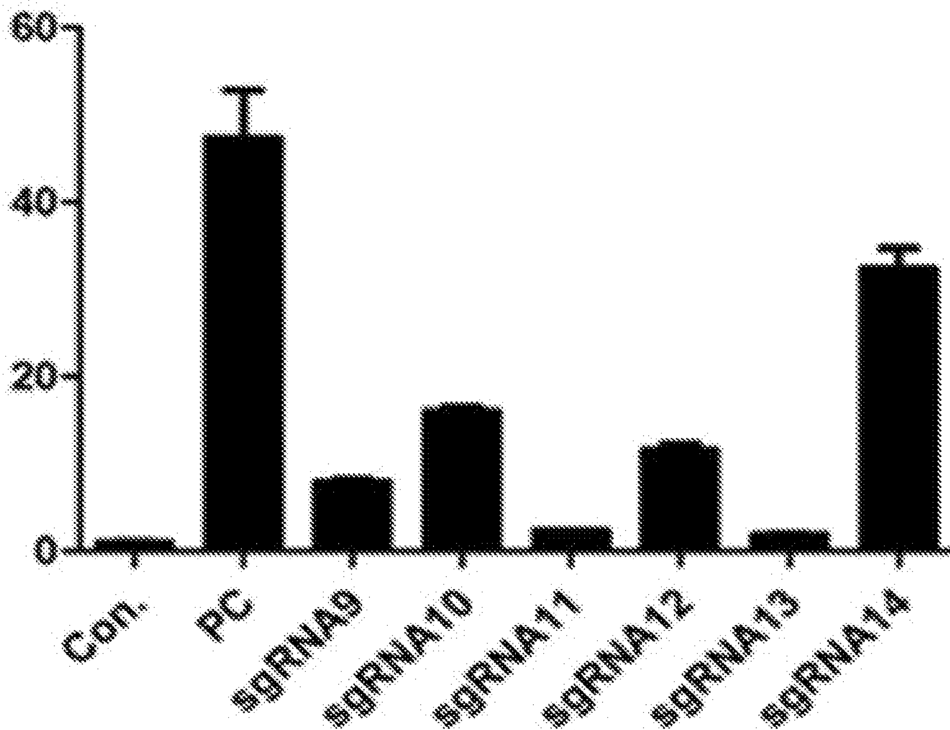


FIG. 1B

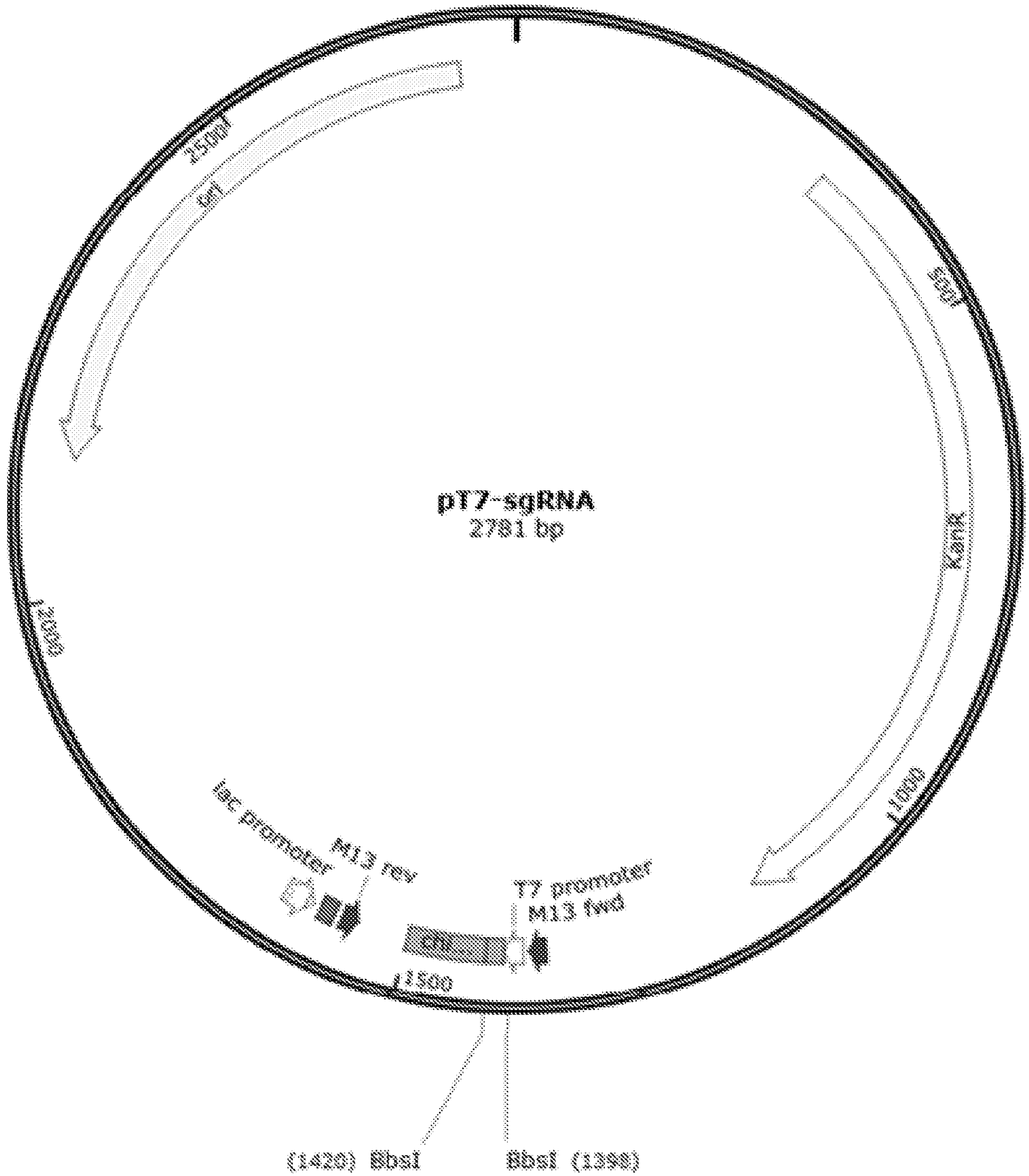


FIG. 2

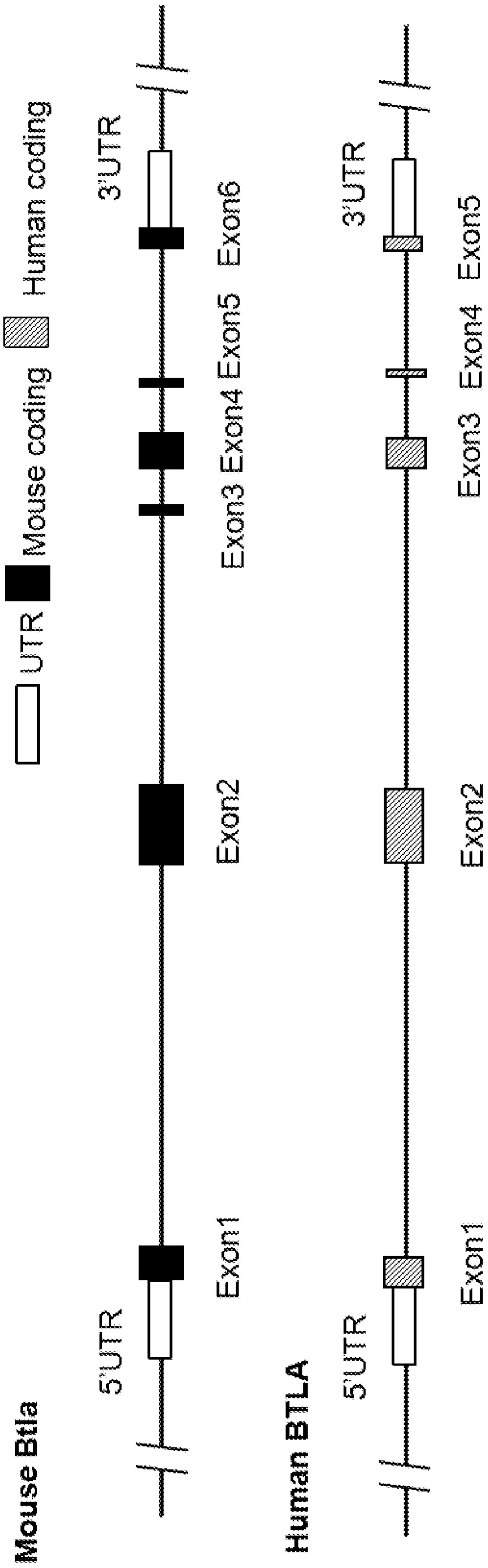


FIG. 3A

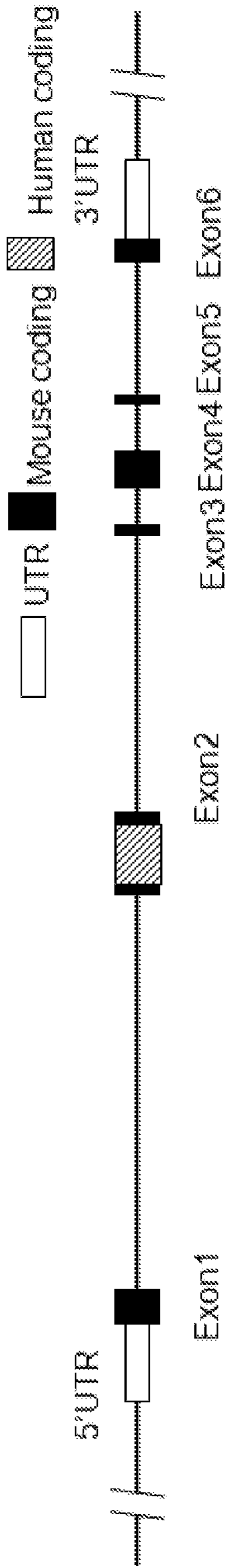


FIG. 3B

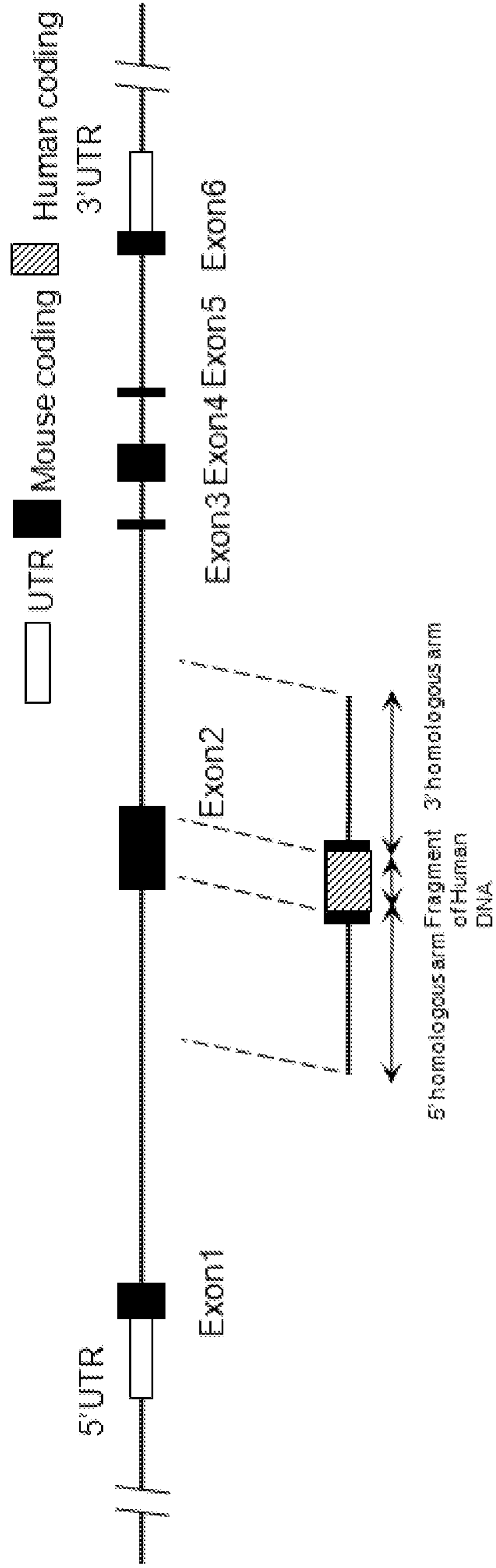


FIG. 3C

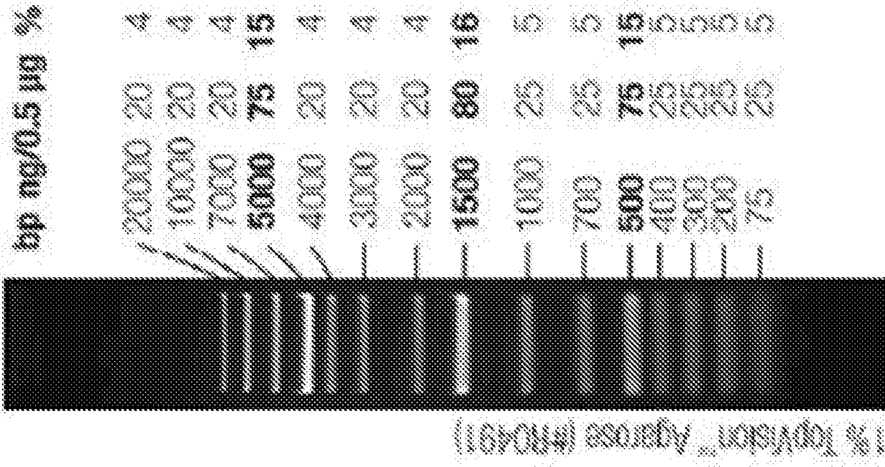


FIG. 4B

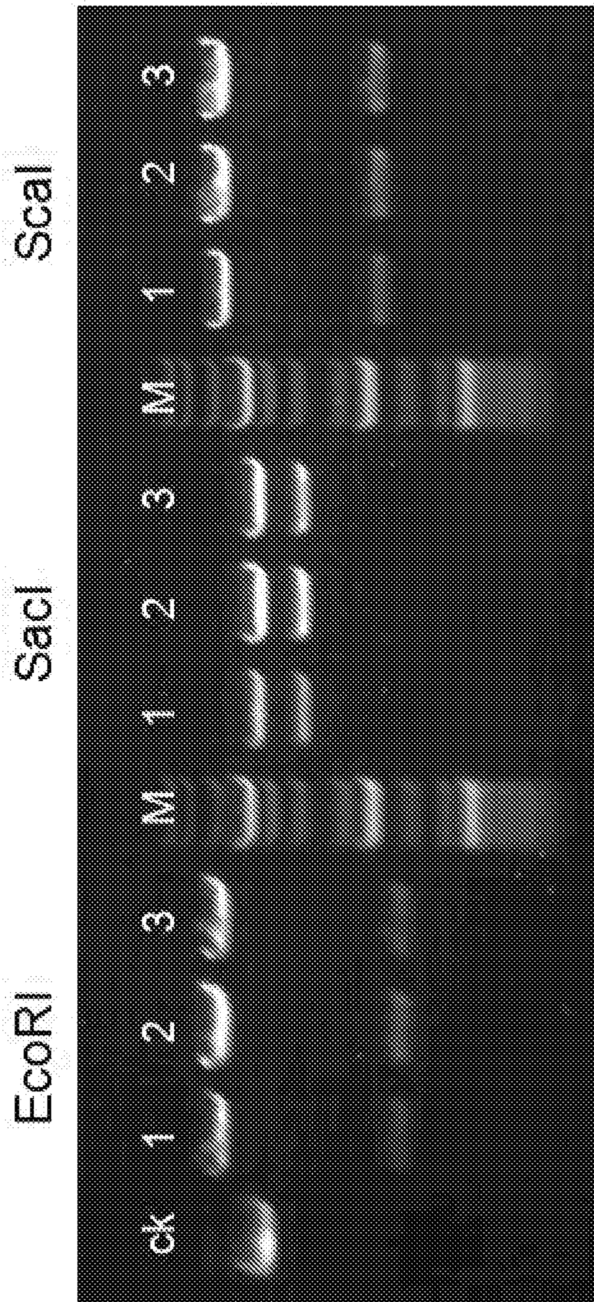


FIG. 4A

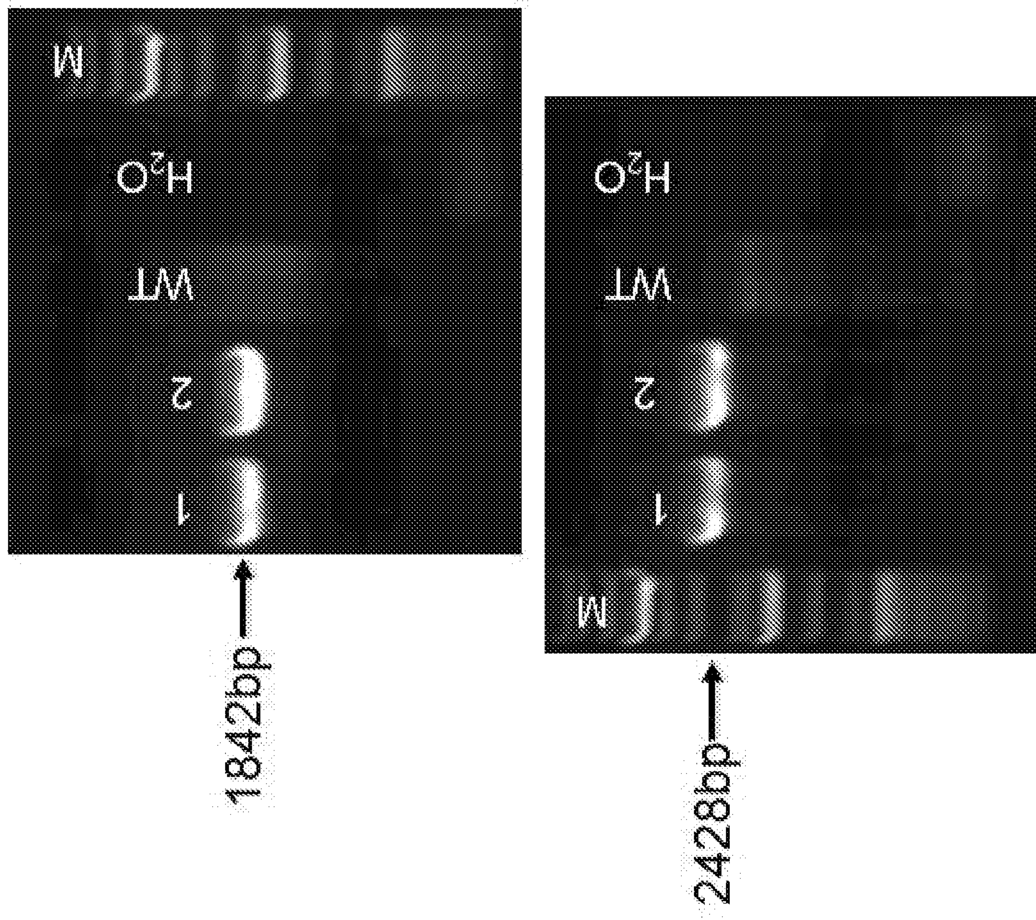


FIG. 5

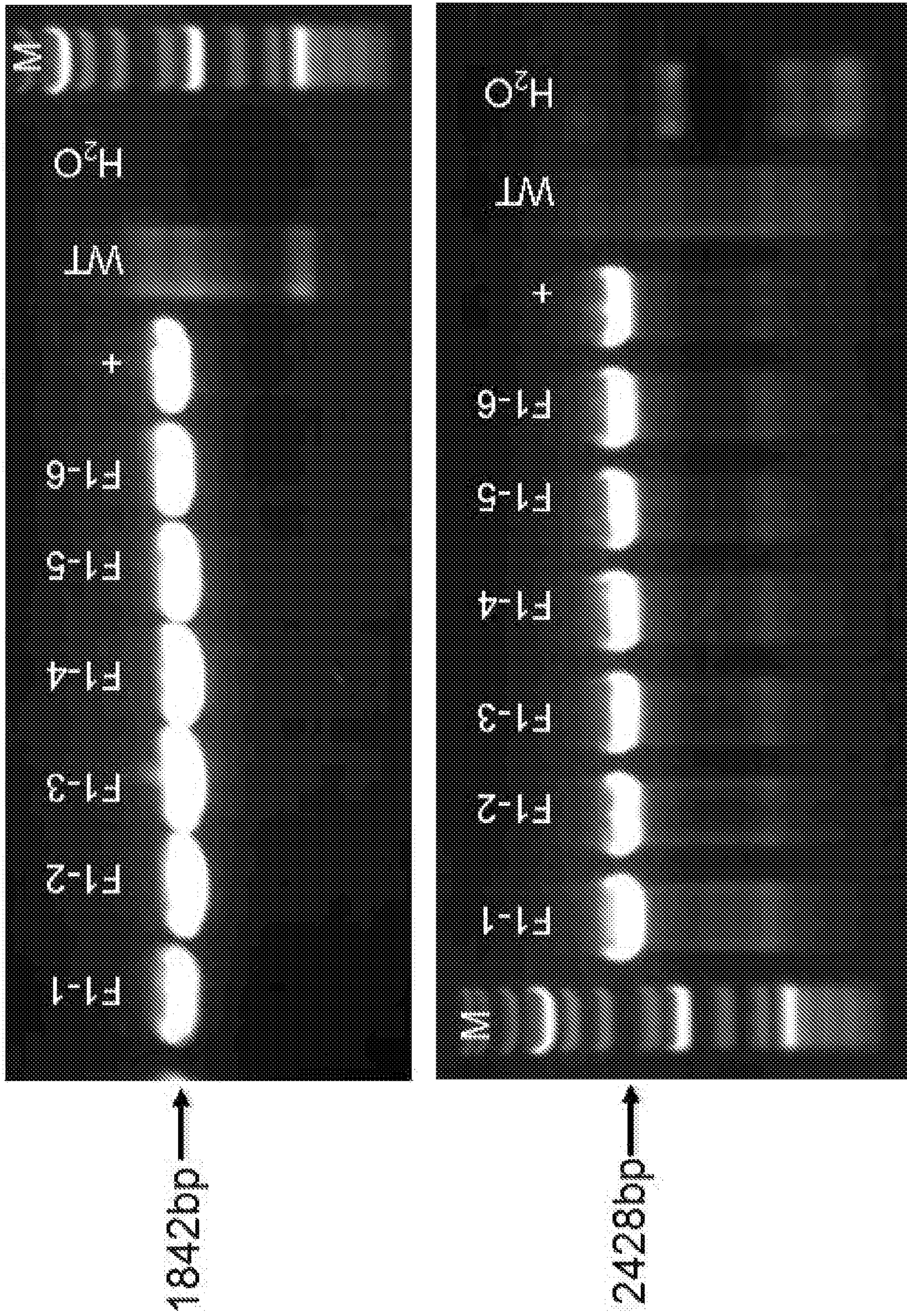


FIG. 6

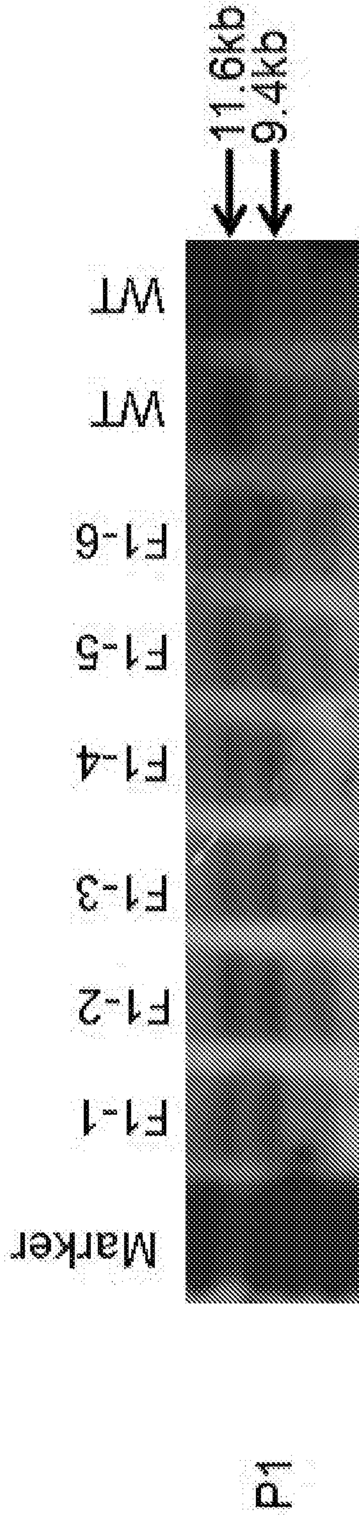


FIG. 7A

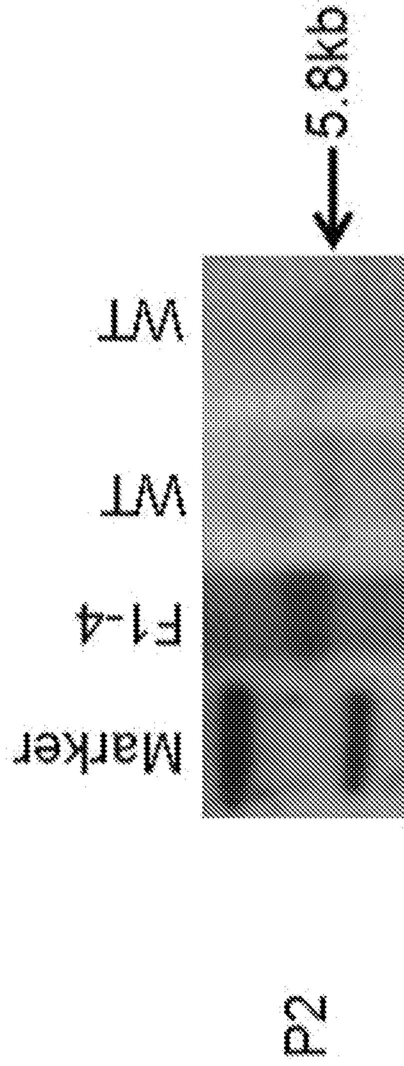
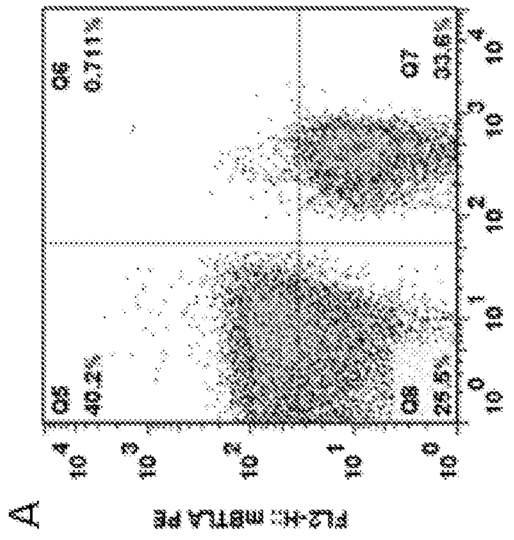
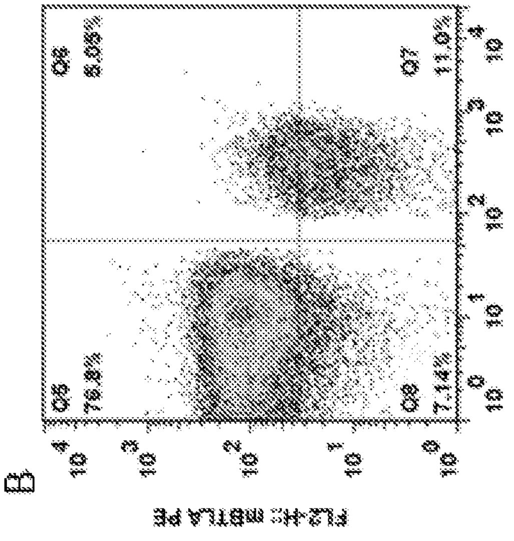
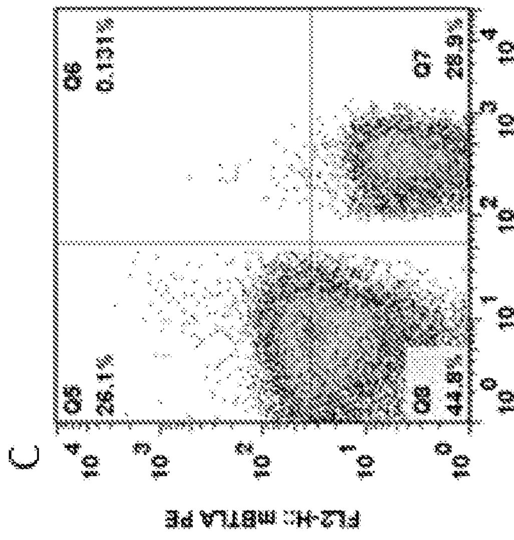
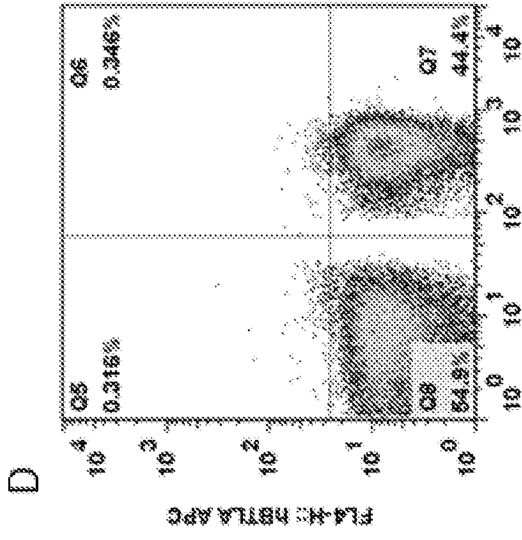


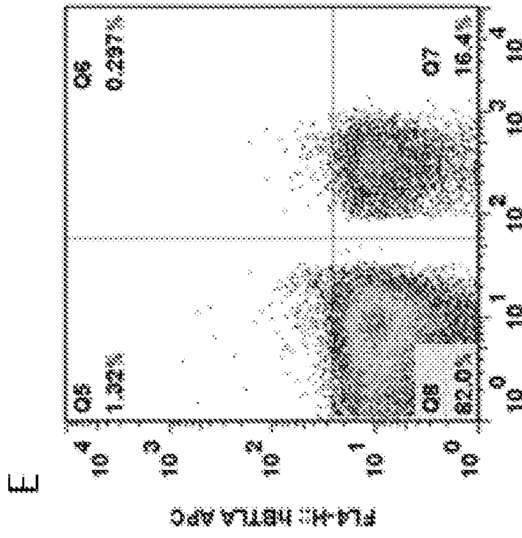
FIG. 7B

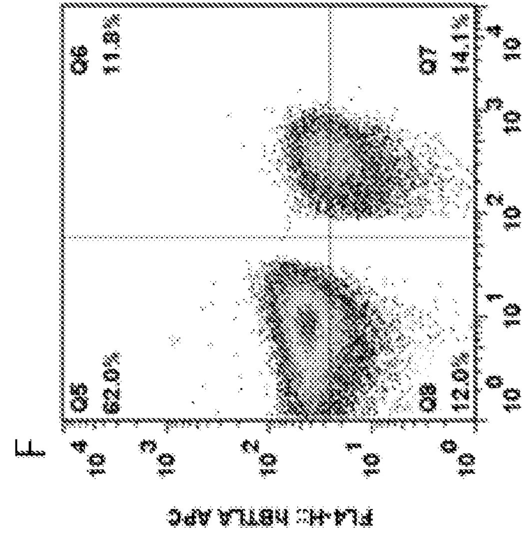












FIGS. 8A-8F

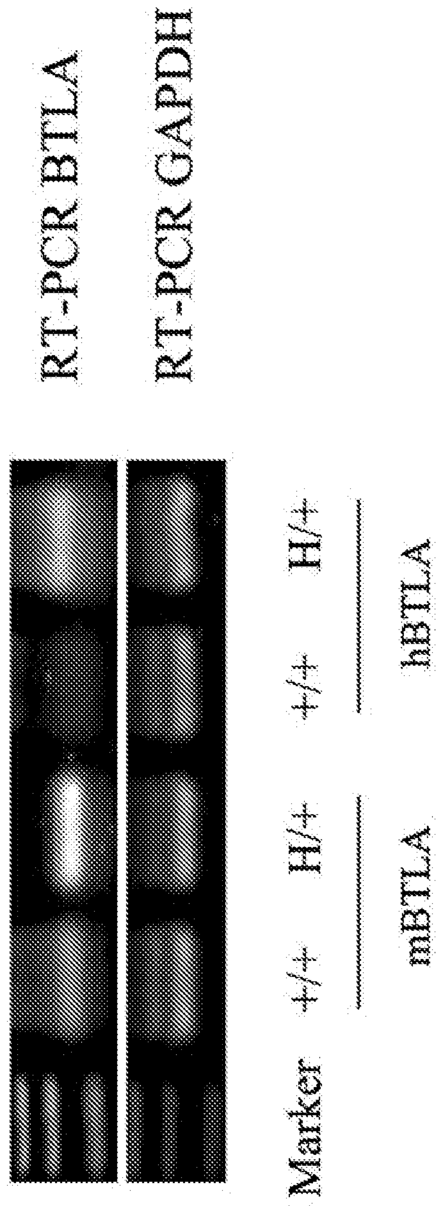
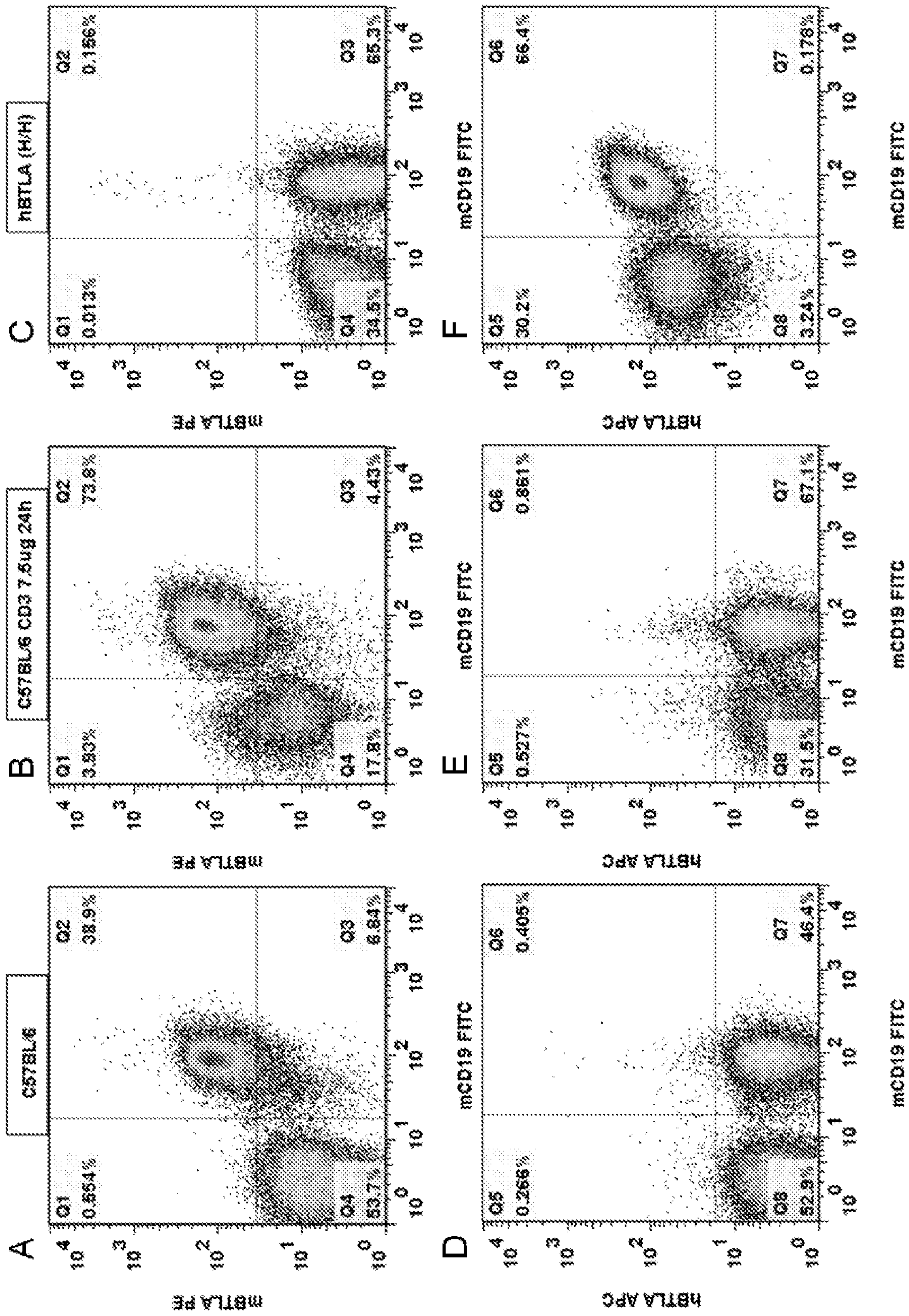


FIG. 9



FIGS. 10A-10F

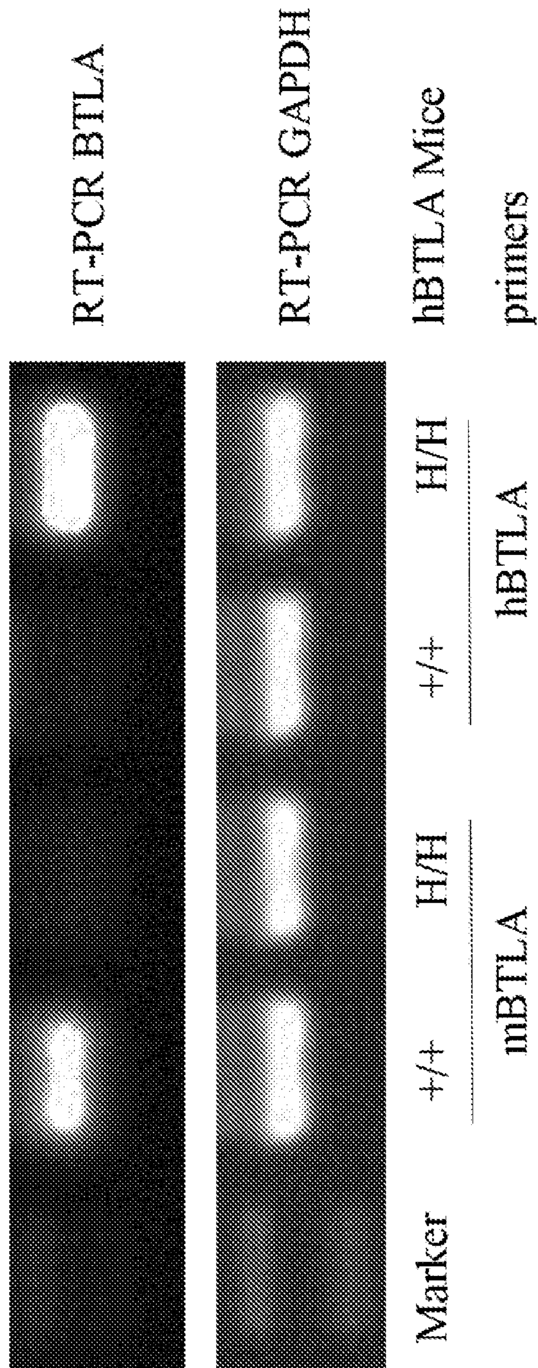


FIG. 11

M 1 2 3 4 5 6 + WT H_2O

452bp

~260bp

FIG. 12

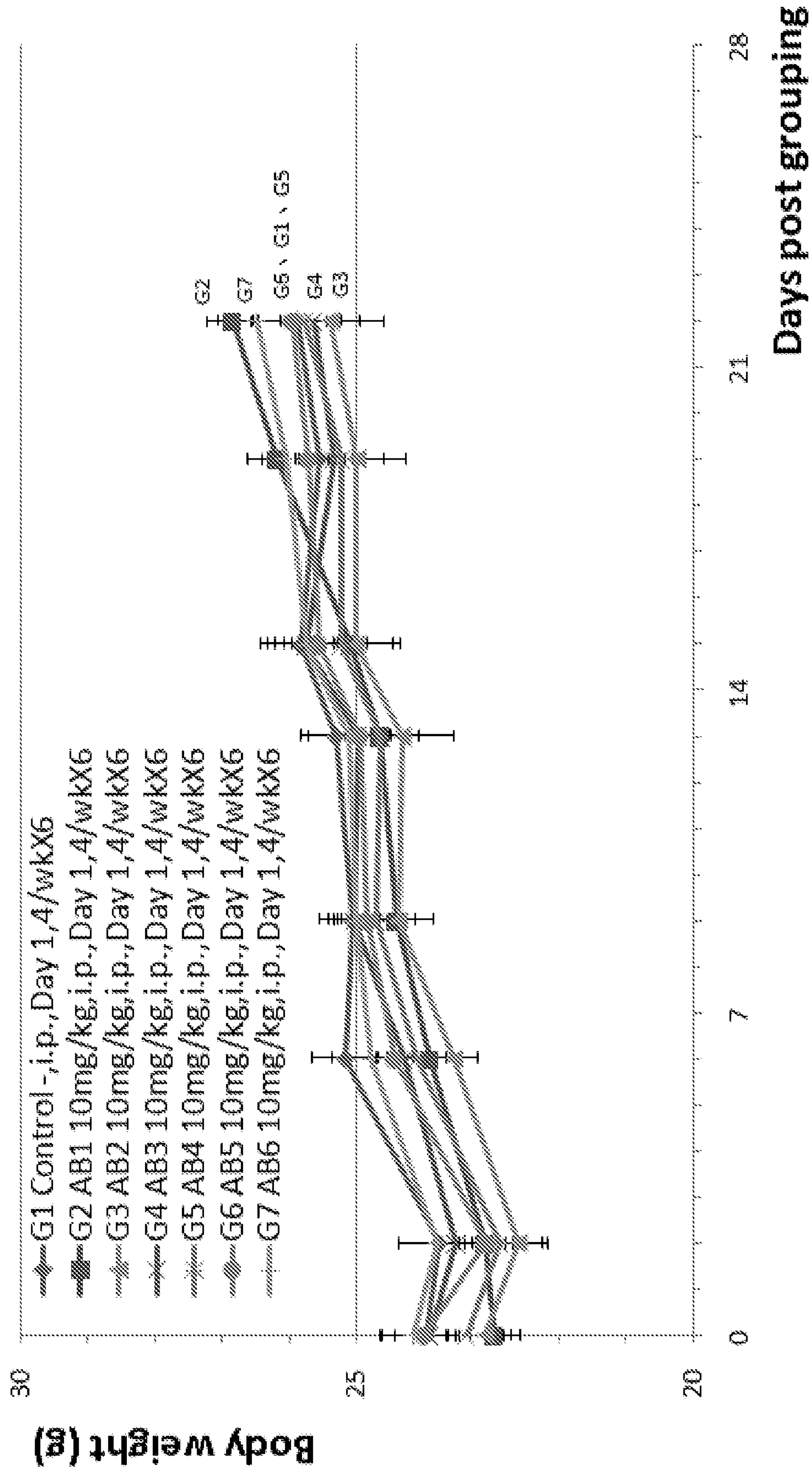


FIG. 13

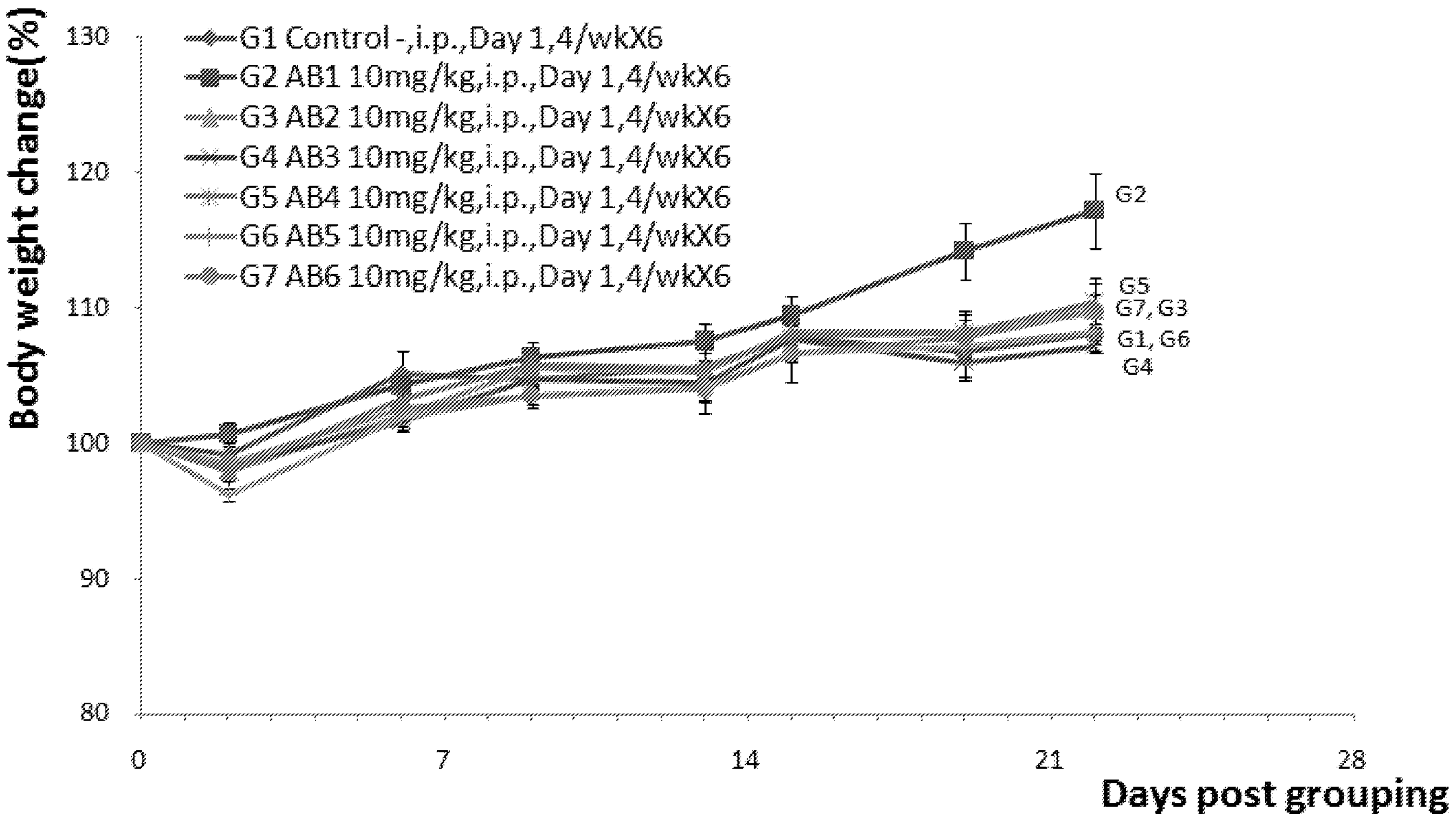


FIG. 14

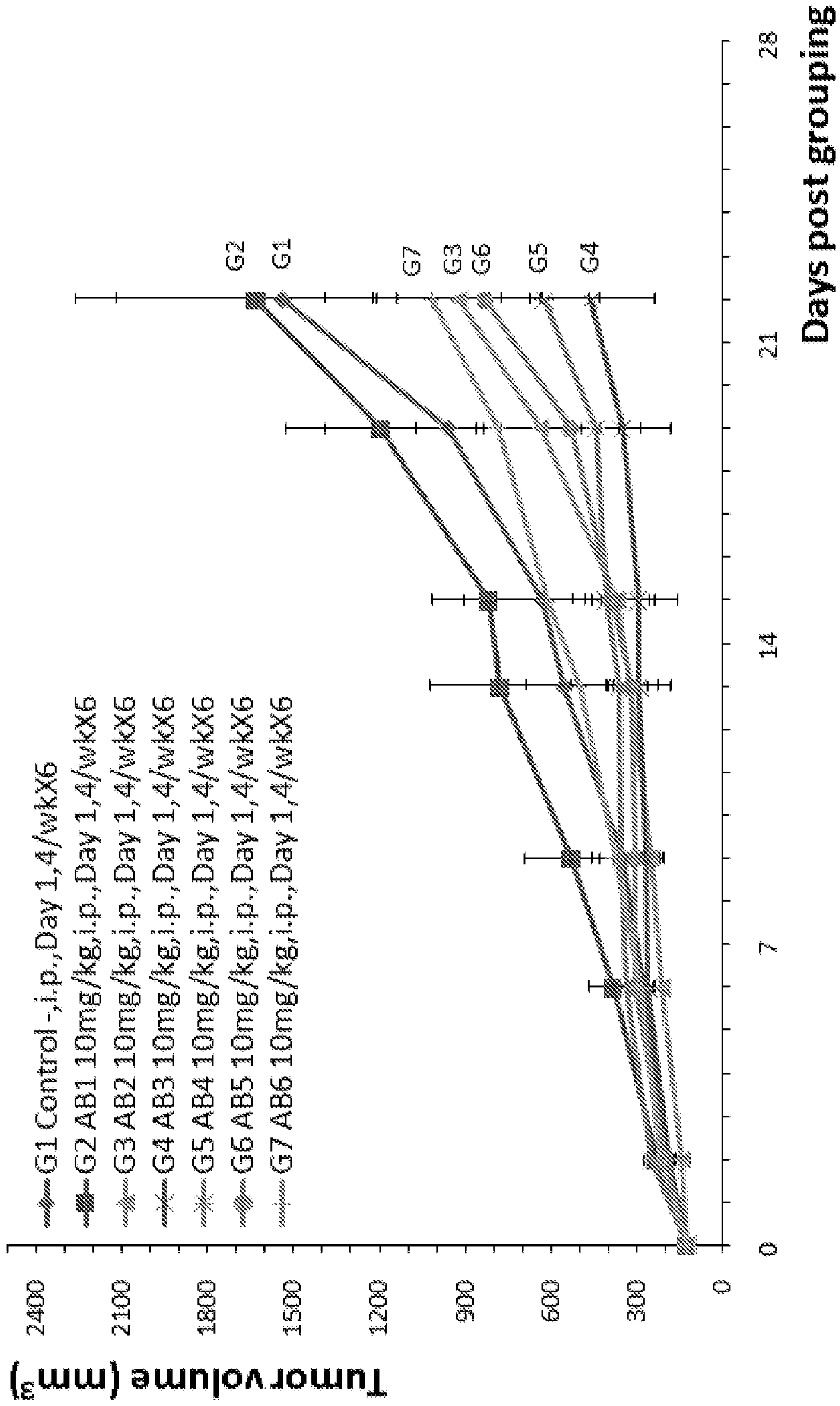
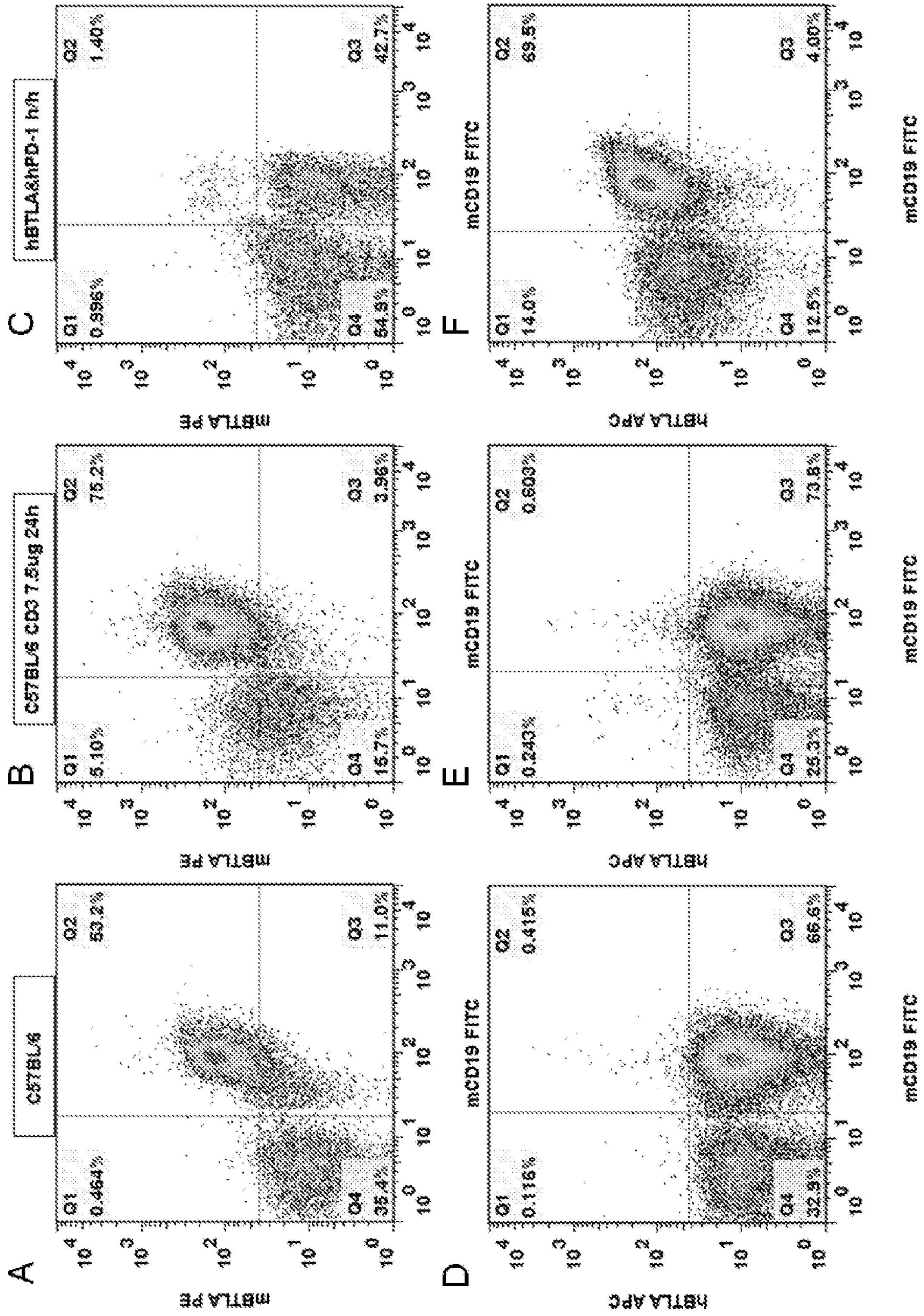
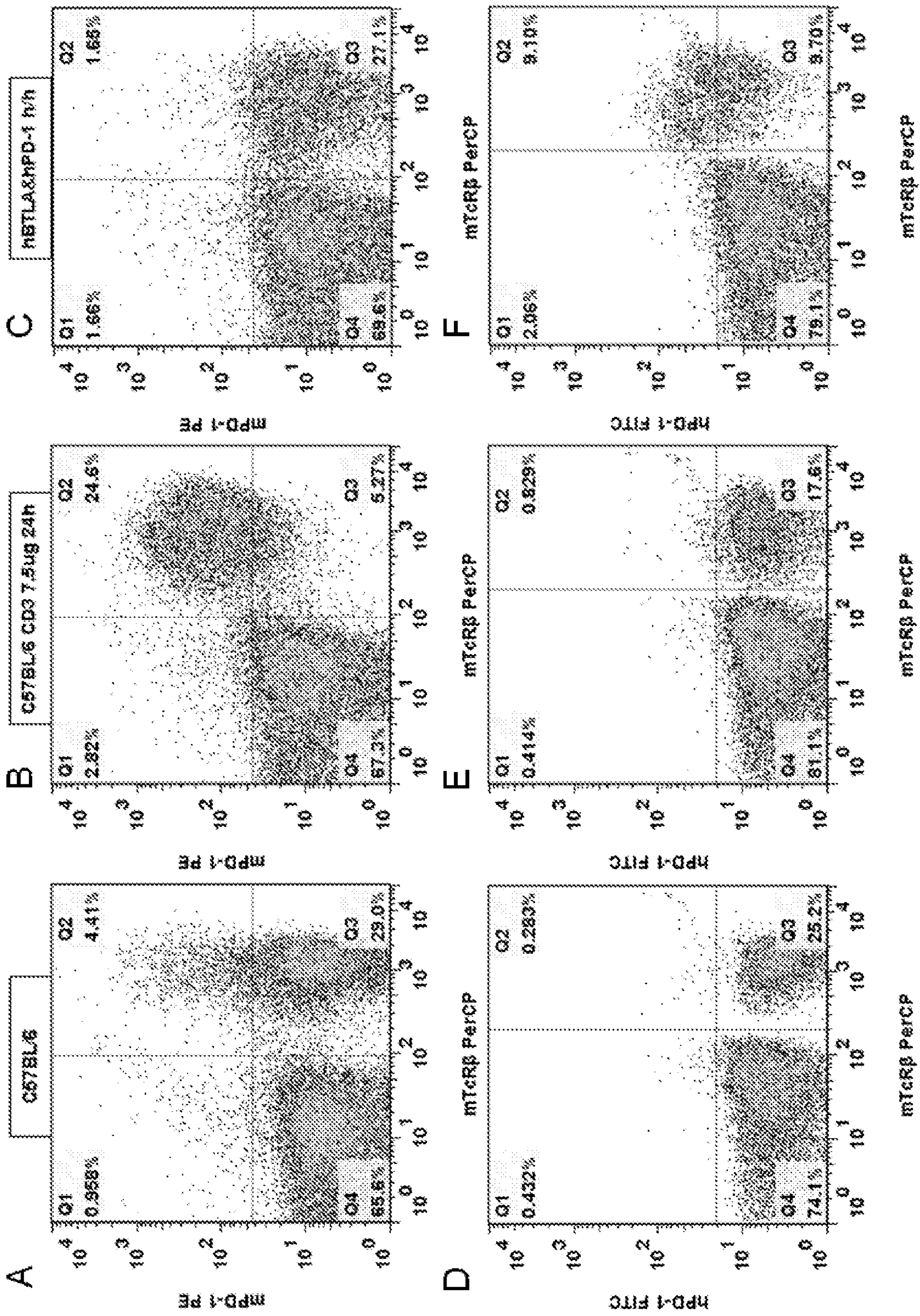


FIG. 15

FIGS. 16A-16D



FIGS. 17A-17F



FIGS. 18A-18F

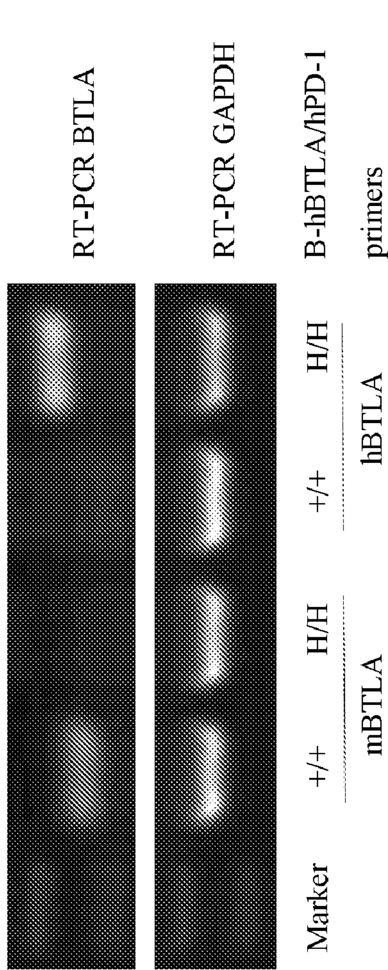


FIG. 19

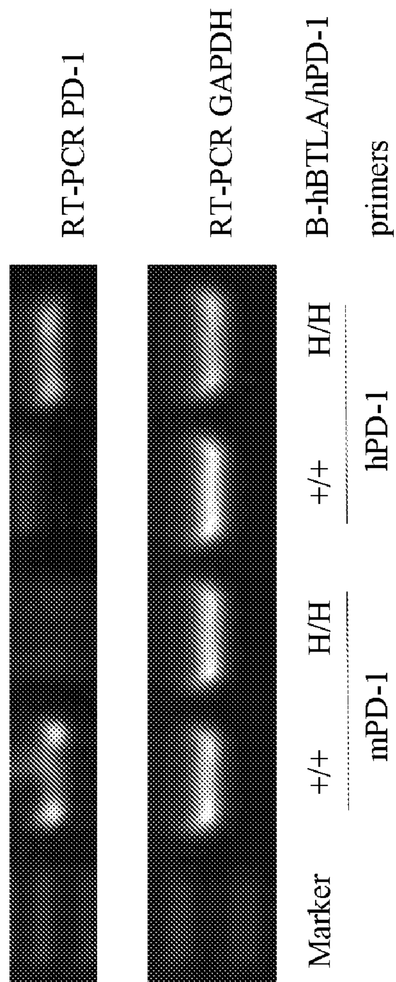


FIG. 20

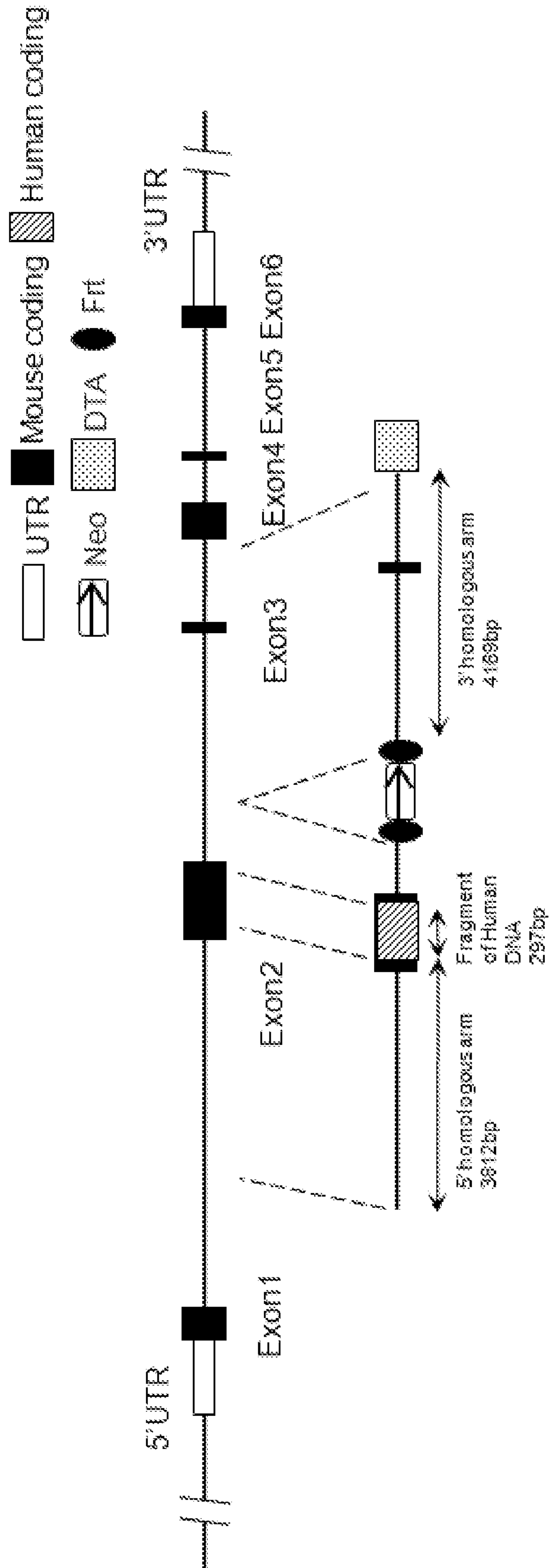


FIG. 21

Score	Expect	Method	Identities	Positives	Gaps
258 bits(658)	5e-90	Compositional matrix adjust.	157/317(50%)	197/317(62%)	39/317(12%)

```
Mouse  1    MKTVPAMLGTPRLFREFFIL-HLGLWSILCEKATKRNDEECPVQLTITRNSKQSARTGEL  59
            MKT+PAMLGT +LF  FF++ +L +W+I          E C VQL I R S+ S   G+
Human  1    MKTLPAMLGTGKLFWVFFLIPYLDIWNI-------HGKESCDVQLYIKRQSEHSILAGDP  53

Mouse  60   FKIQCPVKYCVHRPNVTWCKHNGTICVPLEVSPQLYTSWEENQSVPVFVLHFKPIHLSDN  119
            F+++CPVKYC +RP+VTWCK NGT CV LE      TSW+E +++  F+LHF+P+  +DN
Human  54   FELECPVKYCANRPHVTWCKLNGTTCVKLE---DRQTSWKEEKNISFFILHFEPVLPNDN  110

Mouse  120  GSYSCSTNFNSQVINSHSVTIHVTERTQNSSEHPLITVSDIPDATNASGPSTMEERPGRT  179
            GSY CS NF S +I SHS T++VT                  D  +AS   + +E   R
Human  111  GSYRCSANFQSNLIESHSTTLYVT------------------DVKSASERPSKDEMASRP  152

Mouse  180  WLLYTLLPLGALLLLL-ACVCLLCFLKRIQGKEKKPSDLAGRDTNLVD-------IPASS  231
            WLLY+LLPLG L LL+  C CL C L+R QGK+ + SD AGR+ NLVD         AS+
Human  153  WLLYSLLPLGGLPLLITTCFCLFCCLRRHQGKQNELSDTAGREINLVDAHLKSEQTEAST  212

Mouse  232  RTNHQALPSGTGIYDNDP--WSSMQDESELTISLQSERNNQGIVYASLNHCVIGRNPRQE  289
            R N Q L S TGIYDNDP     MQ+ SE+  +   E N  GIVYASLNH VIG N R
Human  213  RQNSQVLLSETGIYDNDPDLCFRMQEGSEVYSNPCLEENKPGIVYASLNHSVIGPNSRLA  272

Mouse  290  NNMQEAPTEYASICVRS  306
             N++EAPTEYASICVRS
Human  273  RNVKEAPTEYASICVRS  289
```

FIG. 22

INTERNATIONAL SEARCH REPORT	International application No. **PCT/CN2017/106024**

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/85(2006.01)i; C12N 15/90(2006.01)i; C12N 15/113(2010.01)i; C07K 14/705(2006.01)i; C12N 15/12(2006.01)i; A01K 67/027(2006.01)i; A61K 39/395(2006.01)i; A61P 35/00(2006.01)i

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N,C07K,A01K,A61K,A61P

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

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

CPEA, DWPI, JPABS, SIPOABS, VEN, CPRSABS, MOABS, CNABS, TWABS, CJFD, CSCD, SIPONPL USTXT, JPTXT, EPTXT, WOTXT, CNTXT, cnki, isi_web of science:btla,cn272,b7-h4, mouse/mice, transgenic, modified, hvem, c57bl/6; GenBank+EMBL+DDBJ+Retrieving System for Biological Sequence of Chinese Patent:SEQ ID NOs:27,29-31

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Ghazavi F et al. "B- and t-lymphocyte attenuator isoform 1 precursor[Homo sapiens]" *GenBank accession number:NP_861445,* 01 September 2016 (2016-09-01), see the sequence	3, 5, 24, 29
X	Watanabe et al. "Mus musculus B and T lymphocyte attenuator(Btla) mRNA. complete cds" *GenBank accession number:AY293285,* 18 June 2003 (2003-06-18), see the sequence	55-57
X	Breloer M et al. "Mus musculus B and T lymphocyte attenuator(Btla), transcript variant 1, mRNA" *GenBank accession number:NM_001037719,* 01 September 2016 (2016-09-01), see the sequence	55-57
Y	Strusberg, R.L. et al. "Btla protein[Mus musculus]" *GenBank accession number:AAI08965.1,* 04 October 2006 (2006-10-04), see the sequence	4
X	Strusberg, R.L. et al. "Btla protein[Mus musculus]" *GenBank accession number:AAI08965,* 04 October 2006 (2006-10-04), see the sequence	54-57

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
10 January 2018	**19 January 2018**

Name and mailing address of the ISA/CN	Authorized officer
STATE INTELLECTUAL PROPERTY OFFICE OF THE P.R.CHINA **6, Xitucheng Rd., Jimen Bridge, Haidian District, Beijing 100088** **China**	**WANG,Qiyang**
Facsimile No. **(86-10)62019451**	Telephone No. **(86-10)62088409**

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2017/106024

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CN 101578296 A (MEDAREX INC) 11 November 2009 (2009-11-11) see claims 1-87	1-2，6-23，25-28，30-53
Y	CN 101578296 A (MEDAREX INC) 11 November 2009 (2009-11-11) see claims 1-87	3-5，24，29

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:

   a. (means)
      - ☐ on paper
      - ☑ in electronic form

   b. (time)
      - ☐ in the international application as filed
      - ☑ together with the international application in electronic form
      - ☐ subsequently to this Authority for the purposes of search

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

3. Additional comments:

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☑ Claims Nos.: **41-53**
because they relate to subject matter not required to be searched by this Authority, namely:

[1] The subject matter of claims 41-53 relates to a treatment method of the human or animal body (Rule 39.1(iv)), but the search has been carried out and based on the use of the anti-BTLA antibody or the composition consisting of the anti-BTLA antibody and an additional therapeutic agent in the manufacture of medicaments for treating cancer or tumor.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2017/106024

Patent document cited in search report			Publication date (day/month/year)	Patent family member(s)			Publication date (day/month/year)
CN	101578296	A	11 November 2009	WO	2008076560	A3	16 October 2008
				NO	20092285	A	17 August 2009
				US	2012288500	A1	15 November 2012
				AU	2007334264	A1	26 June 2008
				NZ	577085	A	29 June 2012
				ZA	200903775	B	28 April 2010
				ZA	200903775	A	28 April 2010
				CL	2007003291	A1	04 July 2008
				IL	198729	D0	01 August 2011
				US	2010172900	A1	08 July 2010
				AR	063840	A1	25 February 2009
				KR	20090088891	A	20 August 2009
				EP	2097447	A2	09 September 2009
				TW	200837081	A	16 September 2008
				US	8247537	B2	21 August 2012
				WO	2008076560	A9	04 December 2008
				EP	2097447	A4	29 December 2010
				EA	200970477	A1	30 December 2009
				JP	2010509920	A	02 April 2010
				MX	2009005189	A	30 June 2009
				WO	2008076560	A2	26 June 2008
				US	8580259	B2	12 November 2013
				CA	2669921	A1	26 June 2008