CHIMERIC ANTIGEN RECEPTOR, AND T CELLS IN WHICH CHIMERIC ANTIGEN RECEPTOR IS EXPRESSED

TECHNICAL FIELD

The present invention relates to a chimeric antigen receptor recognizing a cancer cell surface antigen, and a T cell in which the receptor is expressed. Specifically, the present invention relates to a chimeric antigen receptor with an excellent expression rate, and a T cell in which the receptor is expressed. More specifically, the present invention relates to a chimeric antigen receptor and a T cell in which the receptor is expressed, said chimeric antigen receptor comprising a cancer cell surface antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain, characterized in that the costimulatory domain consists of mutated CD28 or TNFRSF9. Additionally, the present invention relates to a chimeric antigen receptor and a T cell in which the receptor is expressed, characterized in that the antigen binding domain binds to an antigen selected from the group consisting of IL13Rα2, an antigen associated with an angiogenic activity, EGFRvIII, EphA2, αVβ3, and glypican1.

In addition, the present invention relates to the chimeric antigen receptor and the T cell in which the receptor is expressed, characterized in that three glycines are additionally introduced between the antigen binding domain and the hinge region.

Further, the present invention relates to the chimeric antigen receptor and the T cell in which the receptor is expressed, characterized in that the cytoplasmic signaling domain uses a CD3ζ signaling domain of a normal person where extra Glutamine is comprised, not a CD3ζ signaling domain of a Jurkat T cell.

Moreover, the present invention relates to the chimeric antigen receptor and the T cell in which the receptor is expressed, the chimeric antigen receptor comprising any one of the predetermined antigen binding domain, the costimulatory domain and the cytoplasmic signaling domain described above, or all of them.

BACKGROUND FIELD

T cells (hereinafter, in the present specification, this is sometimes referred to as "CAR-T cells") expressing chimeric antigen receptors (hereinafter, in the present specification, this is sometimes referred to as "CARs") mean recombinant T cells in which a gene coding a receptor recognizing a cancer cell surface antigen specifically expressed on the surface of the cancer cell is introduced into the T cell to kill the cancer cell. Dr, Zelig Fshhar, *et. al.*, who is a chemist and an immunologist of the Weizmann Institute of Science in Israel, had succeeded in making T cells provided with chimeric antigen receptors, by obtaining a theory that when T cells with a receptor binding to an antigen specifically expressed in a cancer call is artificially

made, an immune response occurs, targeting only to the cancer cell, so as to kill the cancer cell, and then reported this fact in PNAS in 1989.

However, CAR-T cells produced in the early stage, i.e. the 1st generation CAR-T cells used only CD3ζ as a signaling domain, but its therapeutic effect was insignificant, and also, there was a disadvantage that the duration time was short. Thus, efforts have been made to improve the reactivity of the CAR-T cells, and as a result, 2nd generation CAR-T cells in which a costimulatory domain (CD28 or CD137/4-1BB) and CD3ζ are combined were produced, wherein the number of CAR-T cells present in the body was significantly increased as compared to the number of the 1st generation CAR-T cells. Meanwhile, the 2nd CAR-T cells used one type of the costimulatory domain, and CAR-T cells using two types of the costimulatory domain are referred to 3rd generation CAR-Ts. Most of the recent studies focus on the 2nd generation and 3rd generation CAR-T cells. Meanwhile, regarding methods for treating cancers using the CAR-T cells, there was a report that when cytotoxic T cells transformed to recognize CD19 were injected into three patients with end-stage chronic lymphoid leukemia (CCL), leukemia was completely treated in two of the patients, and the condition was continued for about 10 months (N. Engl J Med 2011; 365:725-733 August 25, 2011, Sic. Transl. Med 2011 Aug 10;3(95):95ra73). The CAR-Ts used herein correspond to the 2nd generation, and use 4-1BB as the costimulatory domain and CD3ζ as the signaling domain. The antigen binding domain of the CAR-T cells recognizes CD19, which is found

on the surface of the leukemia cancer cells, as an antigen.

In addition, there was a report that when patients with acute leukemia were treated by administering CTL019, 27 of the 30 patients had experienced complete remission, 67% of all patients experienced complete remission for 2 years, and 78% of the patients were survived for 2 years. Given that the subject patients were relapsing or refractory patients, this result was very surprising (N Engl j Med 2014; 371:1507-1517, October 16, 2014).

At present, for therapeutic methods using various CAR-T cells, clinical tests on various hematologic cancers such as lymphoma, myeloma, etc. have been conducted, and it is expected that CAR-T will become available as an available medicine in the market. Since cancer treatment using CAR-T cells is a self-derived method, this product could not be mass-produced; however, this is patient-specific treatment, so its therapeutic effect is incomparably higher than the existing anticancer drugs.

[Patent Literature]

Korean Patent Laid-Open No. 10-2013-0124521

[Non-Patent Literature]

Immunol Rev, 2014, 257(1):107-126

N Engl J Med 2014; 371: 1507-1517, October 16, 2014

Science Translational Medicine 18 Feb 20115: Vol. 7, Issue 275, pp. 275ra22

DETAILED DESCRIPTION OF INVENTION

SUMMARY OF INVENTION

The present invention has a task for providing CAR and CAR-T cells with significantly excellent expression rates and therapeutic effects as compared to the conventionally known CAR-T cells. More specifically, the present invention has a task for providing, in the 2nd generation and 3rd generation CAR-T cells, a costimulatory domain capable of being introduced into various CAR-T cells, as the costimulatory domain serving a main role in the function. Furthermore, the present invention has a task for providing various antigen binding domains capable of binding to an antigen expressed on the surface of a specific cancer cell and also capable of forming CAR-T cells. In addition, the present invention has a task for providing a method for improving the expression rates of various CAR-T cells and the therapeutic effects thereof, with a chimeric antigen receptor characterized by having further amino acid sequences that can be additionally introduced between the antigen binding domain and the hinge domain.

MEANS FOR ACHIEVING TASK

As means for achieving the aforementioned tasks, the present invention describes the

following technical idea.

Disclosed is a chimeric antigen receptor and a CAR-T cell in which the receptor is expressed, the chimeric antigen receptor comprising an antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain, characterized by independent comprising a specific costimulatory domain, or a specific antigen binding domain, and a specific amino acid sequence added between the antigen binding domain and the hinge domain, or in that the cytoplasmic signaling domain uses a CD3ζ signaling domain of a normal person where extra Glutamine is comprised, not a CD3ζ signaling domain of a Jurkat T cell, or by comprising a combination thereof.

EFFECT OF INVENTION

The CAR-T cell comprising the antigen binding domain; or the costimulatory domain; or the signaling domain described in the present invention has the effect that the therapeutic efficacy and expression rate are significantly excellent.

BRIEF DESCRIPTION OF DRAWINGS

The current embodiments are illustrated in the drawings for the purpose of exemplifying the present invention. However, it should be noted that the present invention is not limited to the accurate arrangements and methods of the embodiments illustrated in the

drawings.

Figure 1 illustrates a retrovirus vector and transgene representing the main functional elements of one embodiment of the CAR-T cell described in the present invention. Specifically, this figure illustrates a clinical-grade retrovirus vector indicating the expressions of mutated IL13(E11K.R64D.S67D.R107K), human CD8α hinge and human CD8/human CD3 transmembrane domain in order to impart higher antigen affinity than two amino acid substituents (Glu-11, Arg-107) of IL13, and mutant human CD28(RLLH→RGGH), human TNFRSF9, and CD3 zeta signaling domain of healthy person in order to increase the CAR expression rate. This figure is not illustrated in a constant accumulation.

Figure 2 shows that the expression rate is increased after adding three glycines between the antigen binding domain and the hinge region, in order to increase the solubility of a CAR protein so as to increase the expression of the chimeric antigen receptor.

Figure 3 shows that the expression rate is increased when using mutant (RLLH→RGGH) of human CD28, in order to increase the expression of the chimeric antigen receptor of the CAR protein.

Figure 4 shows the result of analyzing transformed T cell using a Flow cytometry

analysis, in order to verify the purity of CAR-T cell and the expression rate of CAR produced by a production process set for clinical test. This figure shows the CAR expression rate and the T cell purity.

Figure 5 shows the comparison of cytotoxicity of T-cells, in which IL13 (Glu-11, Arg-107) where two positions are substituted, and IL13(IL13.E11K.R64D.S67D.R107K) where four positions are substituted are introduced.

Figure 6 shows CAR repression rate-dependent anticancer activity increase in human brain cancer cell line U251 of CAR-T cells consisting of IL13(E11K.R64D.S67D.R107K).TNFRSF9. CD3ζ where four positions are substituted.

Figure 7 shows IFN-γ cytokine production after culturing human brain cancer cell line U251 (IL13Rα2 overexpression), which is a target cell with CAR-T cells consisting of IL13(E11K.R64D.S67D.R107K).TNFRSF9.CD3ζ where four positions are substituted or HUVEC (lack of IL13Rα2 expression) which is a normal cell control, at a ratio of 0.5 : 1 for 19 hours.

Figure 8 shows that a dose-dependent (CAR+T-cell number) *increase* in IFN-γ cytokine secretion, after culturing human brain cancer cell line U251, which is a target cell

with CAR-T cells comprising (IL13.E11K.R64D.S67D.R107K).TNFRSF9.CD3ζ where four positions are substituted.

Figure 9A is an image briefly showing the nude mouse *in vivo* efficacy experiment method using the CAR-T cells comprising IL13(E11K.R64D.S67D.R107K)TNFRSF9.CD3ζ where four positions are substituted; and Figure 9B shows the result of measuring the tumor size of the animal 12 days after the treatment with the CAR-T cell or PBS.

Figure 9C shows the shape, size and weight of the cancer tissue removed from the nude mouse 15 days after the treatment with the CAR-T cell or PBS.

Figure 10A is an image for the removed cancer tissues stained with anti-human CD3 antibody in order to stain human CAR-T or T cell, from the nude mouse 15 days after the treatment with TMZ+PBS (PBS was intravenously administered once, and TMZ and IL-2 were intraperitoneally and intravenously administered once a day for four days).

Figure 10B is an image for the removed cancer tissues stained with anti-human CD3 antibody in order to stain human CAR-T or T cell, from the nude mouse 15 days after the treatment with TMZ+YYB103 (TMZ and CAR-T cell comprising IL13(E11K.R64D.S67D.R107K)TNFRSF9.CD3ζ where four positions are substituted were

intravenously administered once, and TMZ and IL-2 were intraperitoneally and intravenously administered once a day for four days).

Figure 11A is an image for the removed cancer tissues stained with H&E from the nude mouse 15 days after the treatment with TMZ+PBS (PBS was intravenously administered once, and TMZ and IL-2 were intraperitoneally and intravenously administered once a day for four days).

Figure 11B is an image for the removed cancer tissues stained with H&E from the nude mouse 15 days after the treatment with TMZ+YYB103 (TMZ and CAR-T cell comprising IL13(E11K.R64D.S67D.R107K)TNFRSF9.CD3ζ where four positions are substituted were intravenously administered once, and TMZ and IL-2 were intraperitoneally and intravenously administered once a day for four days).

Figure 12 shows the production of anti-angiogenic CAR. Successful anti-cancer CAR therapies require not only antigen-specific CAR-T cells but also accessing CAR-T cells to cancer cells and maintaining CAR-T cell function in the immunosuppressive tumor microenvironment. Therefore, anti-angiogenic CAR was produced. Figure 12A illustrates the retrovirus vector and transgene representing the main functional element of Anti-Angiogenic CAR; Figure 12B shows the result of analyzing transformed cells checking on the cell surface

expression of Anti-Angiogenic CAR using a Flow cytometry analysis.

Figures 13 to 16 show the four kinds of CAR production, targeting a major tumor antigen of various cancers. A of the respective figures illustrates the retrovirus vector and transgene representing the main functional element; B of the respective figures shows the result of analyzing transformed cells checking on the cell surface expression of Anti-Angiogenic CAR using a Flow cytometry analysis.

BEST MODE FOR EMBODIMENT OF INVENTION

The CAR-T cell according to the present invention is a recombinant T cell in which a receptor gene recognizing a cancer cell as an antigen is introduced, wherein the T cell consists of an antigen binding domain recognizing the antigen; a hinge region (or spacer) connecting the antigen binding domain and a transmembrane domain; the transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain.

The antigen binding domain, which is a site where a main signal is delivered and is present outside of a cell membrane, recognizes a cancer cell expressing a particular antigen. Thus, in the cancer treatment using CAR-T cell, the detailed treatment subject is determined by the antigen binding domain. The present invention describes, for example, a chimeric antigen receptor capable of specifically binding to IL13Rα2 overexpressed in glioblastoma,

but the antigen binding domain is not specifically limited thereto. Glioblastoma (GMB) or glioblastoma multiforme is one of the most general brain tumors, which occupies about 12 to 15% of the brain tumors, and this is a general malignant brain cancer. Since this cancer is relatively rare as compared to a colorectal cancer or lung cancer, the studies on the treatment method thereof have not been actively conducted. The cells of glioblastoma are similar to astrocyte, but astrocyte serves as maintaining nerve cells and giving nutrition to nerves and a defense reaction to the damage of brain tissues. It is believed that genomic abnormality of stem cells or immature astrocyte is involved in occurrence and malignization of glioblastoma. For the treatment of glioblastoma, surgery, radiotherapy and chemotherapy are used. As chemotherapy, temozolomide, lomustine and carmustine, etc. are used, and recently, clinical tests such as tumor vaccine treatment and molecular targeted treatment have been conducted. However, there are still no useful therapeutic agents for the treatment of glioblastoma, and particular, although there was an attempt on the treatment of glioblastoma overexpressing IL13Rα2 using CAR-T cells using mutant IL13 where the 11th position is substituted with E11Y, Baylor college of medicine group reported that the therapeutic effect using the CAR-T cells using mutant IL13 where the 11th position is substituted with E11Y was not good *in vivo*. However, the CAR-T cell described in the present invention exhibits the excellent therapeutic effect of glioblastoma.

Sequence of the antigen binding domain binding to IL13Rα2 is identical to SEQ ID

NO. 1, and in particular, mutant IL13 where the 11th, 64th, 67th and 107th positions are substituted with E11K.R64D.S67D.R107K, respectively, is newly described in the present invention. It should be noted, however, that the amino acids substituted in the corresponding positions can be replaced with amino acids having the similar property with the specific amino acids. Thus, the amino acids can be replaced with arginine (R) or histidine (H), instead of lysine (K), in the 11th position; with glutamic acid (E), instead of aspartic acid (D), in the 64th and 67th positions; and histidine (H), instead of lysine (K), in the 107th position.

IL13(E11K.R64D.S67D.R107K) where the four positions are mutated, described in the present invention, and analogue substituted with an amino acid having the same property in the same position have improved antigen affinity.

The antigen binding domain of the present invention can be produced so as to bind to an antigen expressed in various cancer cells as well as IL13Rα2 overexpressed in glioblastoma. For example, an antigen binding domain (SEQ ID NO. 2) capable of binding to an antigen associated with an angiogenic activity; an antigen binding domain (SEQ ID NO. 3) binding to EGFRvIII which is a main tumor antigen of glioblastoma and lung cancer, etc.; an antigen binding domain (SEQ ID NO. 4) binding to EphA2 which is a tumor antigen of glioblastoma, breast cancer, prostate cancer, etc.; an antigen binding domain (SEQ ID NO. 5) binding to αVβ3 which is a resistant marker of carcinoma stemness and receptor tyrosine kinase

inhibitors (RTKIs) such as erlotinib of pancreatic cancer, lung cancer and breast cancer; an antigen binding domain (SEQ ID NO. 6) binding to glypican1 overexpressed in pancreatic cancer, glioblastoma, breast cancer, etc. are described in the present invention. Figures 12 to 16 show the five kinds of CAR production comprising the above antigen binding domains. The respective figures illustrates the retrovirus vector and transgene representing the main functional element and shows the result of analyzing transformed cells checking on the cell surface expression of Anti-Angiogenic CAR using a Flow cytometry analysis.

The further characteristic of the present invention lies in additionally introducing three glycines between the antigen binding domain and the hinge region, in order to increase the solubility of a CAR protein to increase the expression of the chimeric antigen receptor. According to the study of the present inventors, the difference in solubility between the receptor in which three glycine between the antigen binding domain and the hinge region are introduced and the receptor in which three glycine are not introduced showed about 10 times, and this difference leads to a great difference in expression rate with regard to the production of CAR-T cells, and the difference of the expression rate is eventually directly connected to the therapeutic effect. For this reason, this can be deemed to be very advanced technical development. In addition, said three glycine (G) may be substituted with alanine(A), valine(V), leucine(L) or isoleucine(I), which are amino acids having the similar property.

Yet another characteristic of the present invention lies in the use of a specific costimulatory domain. In the CAR-T cells, when an antigen binding domain and an antigen bind to each other, the signal activates a T cell immune response through the cytoplasmic signaling domain (CD3ζ). The costimulatory domain, which is a site where a costimulatory signal is delivered, serves a role in delivering a signal such that the CAR-T cell recognizing a specific antigen binding to the antigen binding domain causes an immune response to help proliferation and persistence in the body longer. Meanwhile, such costimulatory domain is a component which was not present in the first generation CAR-T cell, and the 2nd CAR-T cell uses one costimulatory domain, and the 3rd generation CAR-T cell uses two costimulatory domains. The CAR-T cell improves proliferation and persistence in the body longer. in the costimulatory domain inside the cells over the generations such as 1st generation, 2nd generation, 3rd generation, etc., and thus the cells have been developed in re-combination with genes such that many CAR-T cells against the cancer cell are made in the body even after injecting a small number of cells, and the cells can last in the body for a long time even after injection. That is, in the case of the first generation, there was a limitation of signaling because it comprises only CD3ζ without the costimulatory domain, but in the 2nd and 3rd generations, 4-1BB or OX40, etc. is additionally introduced so as to improve proliferation and persistence in the body longer.

In addition, the present inventors used the costimulatory domain which generates a

mutant in the predetermined position of CD28 such that by the improvement of the expression rate of CAR-T cells, the therapeutic effect can be exhibited even if less CAR-T cells are used, and furthermore, the inventors have found that by using two costimulatory domains in which mutated CD28 and TNFRSF9 are combined, 'proliferation and persistence in the body' is improved, resulting in the increased therapeutic efficacy.

Accordingly, the further technical characteristic of the present invention lies in a chimeric antigen receptor and a CAR-T cell in which the receptor is expressed, the chimeric antigen receptor consisting of an antigen binding domain; a transmembrane domain; a costimulatory domain and a cytoplasmic signaling domain, wherein the costimulatory domain comprises CD28 or TNFRSF9, or CD28 and TNFRSF9. Here, CD28 may comprise a mutant (Sequence Nos. 6 to 9 are substituted from RLLH to RGGH) for increasing the expression of the chimeric antigen receptor. Amino acid sequences of the wild type of CD28 and TNFRSF9, which may be comprised as the costimulatory domain of the present invention, are indicated with Sequence Nos. 7 and 8. In addition, in the mutated CD28 (Sequence Nos. 6 to 9 are substituted from RLLH to RGGH), the substituted amino acid is glycine(G), but this can be replaced with amino acid similar thereto, for example alanine(A), valine(V), leucine(L) or isoleucine(I). The hinge region of the present invention is a portion connecting the antigen binding domain and the transmembrane domain, and this is also called spacer. The hinge region has the purpose for expanding the antigen binding domain from T cell membrane. As

the hinge region of the present invention, a hinge region typically used in the pertinent technical field can be used, and for example, the hinge region can be derived from CD8 hinge region (SEQ ID NO. 9, SEQ ID NO. 10). As aforementioned, another technical characteristic of the present invention lies in three glycines additionally introduced between the antigen binding domain and the hinge region.

The transmembrane domain of the present invention severs a role as an anker of CAR molecular and at the same time a role in delivering a signal received from the antigen binding domain to the costimulatory domain and the cytoplasmic signaling domain. The transmembrane domain is not limited to the transmembrane domain of the present invention, and typical transmembrane domains used for the CAR production can be used, and for example, human CD8/CD3 transmembrane domain can be used (SEQ ID NO. 11, SEQ ID NO. 12).

As the cytoplasmic signaling domain of the present invention, a CD3ζ signaling domain of a normal person where extra Glutamine is comprised, not a CD3ζ signaling domain of a Jurkat T cell, was used, and the extra glutamine means glutamine(Q) in the 50th position in SEQ ID NO. 13.

The technical idea of the present invention includes both the use of the

aforementioned technical characteristics alone and the use of the combination thereof. That is, the implementation of the technical characteristics of the present invention includes all of the CAR-T cells comprising the predetermined antigen binding site described in the present invention; the CAR-T cells in which three glycine are additionally introduced between the antigen binding domain and the hinge region; and the CAR-T cells comprising the costimulatory domain described in the present invention.

The nucleic acid sequences of polypeptide constituting the domains described in the present specification can be obtained using the recombinant methods known in the pertinent technical field, and for example, can be obtained by screening library from cells expressing the gene using the standard technique, or inducing a gene from a known vector to comprise the same gene, or directly isolating from cells or tissues comprising the same gene. Alternatively, the interested gene can be generated by synthesis, not cloning.

The method for introducing and expressing a gene into cells has been known in the pertinent technical field. The expression vector can be rapidly introduced into host cells by any method known in the pertinent technical field. For example, in the present invention, the CAR-T cells can be produced by joining a CAR gene fragment finally produced to an MFG retrovirus expression vector cleaved with XhoI/NotI. It should be understood that the present invention comprises any various mutants for each of the components of the structure.

Cancers to be treated may include non-solid tumors (for example, hematological tumors, e.g. leukemia and lymphoma) as well as glioblastoma, or may include solid tumors.

As such, it takes several steps to produce recombinant CAR-T cells and inject the cells into cancer patients. T cells are isolated from the blood of patients, and then DNA designed with CAR is introduced into the T cells using an expression vector, and the CAR-T cells are proliferated, and then injected back into the patient.

MODE FOR CARRYING OUT THE INVENTION

Hereinafter, the present invention is specified through the examples. Please be noted, however, that the following examples are not intended to limit the technical scope of the present invention in any meaning.

Example 1: Construction of an expression vector having 2nd generation (YYB-103, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) and 3rd generation (YYB-103A, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) chimeric antigen receptors specifically binding to IL13Rα2 overexpressed in cancer cell

The present inventor produced mutant IL13(E11K.R64D.S67D.R107K) in order to impart a higher antigen affinity than two amino acid substitutions (Glu-11, Arg-107) of IL13,

and produced chimeric antigen receptors comprising one co-stimulation domain (TNFRSF9) and two co-stimulation domains (CD28, TNFRSF9) of the cytoplasmic signaling domain of CAR-T cell (Figure 1). In the present invention, three glycines were added between the antigen binding domain and the hinge region in order to increase the solubility of a CAR protein to increase the expression of the chimeric antigen receptor (Figure 2). The CD28 cytoplasmic signaling domain used in the present invention comprises mutant (RLLH→RGGH) human CD28 DNA sequences for increasing the solubility of a CAR protein to increase the expression of the chimeric antigen receptor (Figure 3). Human CD3ζ signaling domain of healthy normal person, not a CD3ζ signaling domain of a Jurkat T cell, was used. Human IL13(P35225.1), human CD3(P20963-1), human CD8α(P01732), human CD28(P10747), human TNFRSF9(Q07011), and human kappa light chain signal sequence (HuVHCAMP) were optimized using science literatures and publicly available database, the 2nd and 3rd generation chimeric antigen receptors (YYB-103, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ and YYB-103A, IL13.E11K.R64D.S67D.R107K.28.TNFRSF9.CD3ζ) consisting of codon-optimized synthetic DNA was produced (Figure 1). The completed structure comprises kozak consensus ribosome-binding sequence, human kappa light chain signal sequence (HuVHCAMP), human IL13.E11K.R64D.S67D.R107K mature protein sequence (human IL13(E11K.R64D.S67D.R107K)), an addition of three glycine (GGG) between the antigen binding domain and the hinge region in order to increase the solubility of a CAR protein to

increase the expression of the chimeric antigen receptor (Figure 2), the hinge region of human CD8α, human CD8/CD3 transmembrane domain, the costimulatory domain of cytosol CD28 transformed to mutant (RLLH→RGGH), the costimulatory domain of cytosol TNFRSF9, CD3ζ cytoplasmic signaling domain of healthy person and Xhol/NotI cleavage portion. The entire sequences of YYB-103 and YYB-103A are represented in Sequence Nos. 14 and 15. A CAR gene fragment finally produced was joined to an MFG retrovirus expression vector cleaved with XhoI/NotI (Emtage PC, etc., Clin Cancer Res, 2008 14L8112-8122) (Figure 1). In the present example, in order to compare the activity of chimeric antigen receptors, two amino acid substitutions (Glu-11, Arg-107), 3rd generation chimeric antigen receptors (YYB-103B, IL13(E11K.R107K).28.TNFRSF9.CD3ζ) of IL13 was additionally produced (SEQ ID NO. 16).

Example 2: Production of T cells transformed to 2nd generation (YYB-103, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) and 3rd generation (YYB-103A, IL13.E11K.R64D.S67D.R107K.28.TNFRSF9.CD3ζ) chimeric antigen receptors specifically binding to IL13Rα2 overexpressed in cancer cells.

PG13 clone with high titer, expressing CAR, was produced by temporarily infecting phoenix-empo and phoenix-eco cells with the retrovirus expression vector YYB-103 or YYB-103A produced in Example 1, and subsequently transduced PG13 cells with acellular vector

stock from the infected phoenix-empo and phoenix-eco cells. Single clone with high titer was obtained by a flow cytometer after staining PG13/YYB-103 cells using an anti-IL-13 monoclonal antibody (BD Pharmingene). PG13/YYB-103 single clone with high titer was produced by the second subcloning according to the limit dilution assay. Subclone PG13/YYB-103-13 showed the stably high CAR expression, and was selected to transduce peripheral blood mononuclear cells (PBMC) of human. The transduction efficiency of the PBMC/YYB-103-13 checked with anti-iL-13 monoclonal antibody (BD Pharmingen) using a flow cytometry analysis. The supernatant of PG13/YYB-103-13 cells comprises retrovirus and collected for the genetic modification of PBMC. PBMC were separated using centrifugation by putting whole blood obtained from a healthy human donor into Ficoll Paque (GE Healthcare). The separated PBMC was cultured by adding anti-CD3 monoclonal antibody (eBioscience) 100ng/mL under the condition of Human IL-2 (NOVARTIS) 100IU/mL to activate the T cell fraction (BL Levine, Cancer Gene Therapy, 2015, 22:79-84). After 2 to 3 days activation, most of the cells were T cells, and comprised natural killer cells at a ratio of 0-2%. After 2 to 3 days activation step, the T cells were subject to transduction two times over 2 days using retroviral supernatant and were washed, and then the cells were proliferated for 4 to 7 days in a flask. The cells were cultured in a stirring platform device (WAVE bio-reactor system) for 12 to 14 days. IL-2 was maintained in the amount of 100IU/mL. The CAR-T cell modified in such manner was used for the analysis experiment (Figure 4).

Experimental Example 1: Check for the CAR expression rate of the T cell surface transformed to a chimeric antigen receptor

Experimental method (flow cytometric analysis)

For flow cytometry (>30,000 events), BD LSRII equipment (Becton Dickinson) and BD FACSDiva software (Becton Dickinson) were used. Specifically, before adding a PE-conjugated anti-human IL-13 monoclonal antibody (BD Pharmingen), the cell was washed once with PBS containing 2% bovine serum albumin. After washing, the cell was reacted with the respective antibodies for 30 minutes at 4℃ in the state where light was blocked and then washed once, and thereafter, the expression rate of the transduced T cell surface CAR was checked. In addition, in order to verify the cell surface expression of IL13Rα2 and IL13Rα1, anti-human IL13Rα antibody (R&D systems), donkey anti-goat IgG phycoerythrin secondary antibody (R&D systems), and anti-human IL13Rα phycoerythrin (R&D systems) were used, and as a control, isotype antibody was comprised.

Experimental result

In order to verify that IL13Rα2-specific CAR(YYB-103, IL13.E11K.R107K.TNFRSF9.CD3ζ; YYB-103A, IL13.E11K.R64D.S67D.R107K.28.TNFRSF9.CD3ζ;YYB-103B, IL13.E11K.R107K.28.TNFRSF9.CD3ζ) produced in Example 1 was expressed on the T cell

surface, T cell cultivation was carried out for 12 - 14 days according to Example 2 and then the flow cytometric analysis was carried out according to the experimental method. As the result of the analysis, the expression rates of the chimeric antigen receptors expressed on the living T cell surface were 90.5% to 92.8% in the seven (7) blood donors. When the cultivation was maintained, the expression of IL13Rα2-specific chimeric antigen receptor was stably maintained for 4 weeks, without additional T cell activation or transduction. In addition, in the cultured cell, the ratios of entire T cell, CD4 T cell, CD8 T cell, B cell, and Monocyte were analyzed. As the result, it was confirmed that B cell existed in the amount of 0.5-1.2%, and no Monocyte exists.

Example 3: Measurement of cytotoxicity and IFN-γ secretion of the T cell transformed to 2nd generation (YYB-103, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) and 3rd generation (YYB-103A, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) chimeric antigen receptors specifically binding to IL13Rα2 overexpressed in cancer cell

IL13Rα2-specific cytotoxicity and IFN-γ secretion were measured using the CAR-T cell produced in Example 2. In order to measure IL13Rα2-specific cytotoxicity and IFN-γ secretion, U251 that is a glioblastoma human cell line overexpressing IL13Rα2, and primary HUVEC that does not express IL13Rα2, as a normal cell control, were used.

Experimental Example 1: Cytotoxicity check for glioblastoma overexpressing

IL13Rα2

Experimental method

In order to measure cytotoxicity of IL13Rα2-specific CAR-T cell effector (IL13Rα2-specific CAR+ T cell effector), cytotoxicity assay was carried out using a DELFIA (Perkin Elmer) kit. Specifically, CAR-T cell effector cells were used 12 to 14 days after the cell activation to anti-CD3, and the experiment was carried out three times under the condition where the effector cell was put in a 96 well plate in which IL13Rα2 target cell existed at a ratio of 5:1 (effector:target) and a ratio of 0.625:1 (effector:target) and reacted for 2 hours at 37℃. In the 96 well plate used in this analysis experiment, 5,000 target cells were added per well, and U251, which is a glioblastoma cell line overexpressing IL13Rα2, was used as the used target cell, and primary HUVEC was used as a normal cell control.

Experimental result

It was analyzed whether the target cancer cell (U251) overexpressing IL13Rα2 was effectively killed by IL13Rα2-specific CAR(YYB-103, IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ;YYB-103A, IL13.E11K.R64D.S67D.R107K.28.TNFRSF9.CD3ζ; YYB-103B, IL13.E11K.R107K.28.TNFRSF9.CD3ζ) T cells produced according to the present invention. As the experimental method, the method of comparing and analyzing cytotoxicity by culturing the target cancer cell (U251) and the normal cell (HuVEC) aforementioned together with the

respective activated CAR-T cell was used. As illustrated in Figure 5, in all cases, the result was obtained that when CAR specific to IL13Rα2 was expressed, the CAR expression induced the killing of glioblastoma U251 cell expressing IL13Rα2 at a high level, as compared to the activated T cell that was not transduced (Figure 5). Specifically, in the case of the T cell expressing YYB-103(IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ), YYB-103A(IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ), and YYB-103B(IL13.E11K.R107K.28TNFRSF9.CD3ζ) CAR produced in Example 2 of the present invention, as the E:T ratio increased, cytotoxicity gradually increased; however, in the case of T cell that did not express CAR, cytotoxicity rarely increased. In addition, as the result of comparing YYB-103B(IL13.E11K.R107K.28.TNFRSF9.CD3ζ) CAR, in which two amino acids of IL13 were substituted, and YYB-103 (IL13.E11K.R64D.S67D.R107K.TNFRSF9.CD3ζ) and YYB-103A(IL13.E11K.R64D.S67D.R107K.28.TNFRSF9.CD3ζ), in which four amino acids of IL13 were substituted, which were used in order to impart higher antigen affinity, in the case of YYB-103B in which only two amino acids were substituted, 67.4% of cytotoxicity was shown at the E:T ratio of 5:1, but when YYB-103 and YYB-103A in which mutant IL13.E11K.R64D.S67D.R107K in which four amino acids of IL13 were substituted were used, 85.6% and 87.7% of cytotoxicity were shown, respectively. This shows that cytotoxicity of T-cell in which mutant IL13.E11K.R64D.S67D.R107K in which four amino acids of IL13 were substituted was introduced is superior in both 2nd generation and 3rd generation as

compared to cytotoxicity of T-cell in which mutant IL13.E11K.R107K in which two amino acids of IL13 were substituted was introduced.

From the experimental result of using, as a normal cell control, HUVEC cell which does not express IL13Rα2 but minimally expresses IL13Rα1, as an object to be compared with the target cells, it was confirmed that the CAR-T cell (12.3-14% cytotoxicity) specific to IL13Rα2 has very weak cytotoxicity (Figure 5). This shows that due to the property of the HUVEC cell which expresses IL13Rα1 and does not express IL13Rα2, the chimeric antigen receptor used in the experiment specifically binds to IL13Rα2. Through the present experimental example, it was demonstrated that IL13Rα2-specific chimeric antigen receptor T cell does not exhibit toxicity to a normal cell (HUVEC) and can significantly kill the target cancer cell (U251) expressing IL13Rα2.

Experimental Example 2: Measurement of anticancer activity change according to CAR expression rate

Experimental method

In order to measure the anticancer activity change according to the expression rate of IL13Rα2-specific CAR, the cytotoxicity analysis was carried out. Specifically, 2nd Generation IL13.E11K.R64D.S67D.R107K.TNFSFR9.CD3ζ, which is YYB-103, was used as an effector cell for CAR-T cell, and U251 cell line overexpressing IL13Rα2 was used as a

Target cell. For the cytotoxicity analysis, the ratio of the effector cell and the target cell was 0.625:1 and 5:1, and the ratio of T-cell expressing CAR was 0-70%. The detailed method of the cytotoxicity analysis is the same as the experimental method of Experimental Example 2.

Experimental Result

In case where T cell expressing IL13Rα2-specific CAR did not exist, 16.4% and 2.5% of cytotoxicity were shown respectively at the ratio of 5:1 and 0.625:1. However, it can be shown that as the ratio of the T cells expressing IL13Rα2-specific CAR increased, the cytotoxicity increased, and it was demonstrated that when the ratio of the T cells expressing IL13Rα2-specific CAR was 70%, 86% and 26% of the cytotoxicity were shown respectively at the ratios of 5:1 and 0.625:1, and thus IL13Rα2-specific chimeric antigen receptor T cell can kill IL13Rα2-specific target cancer cell (U251) (Figure 6).

Experimental Example 3: Check for the production of cytokine (IFN-γ) of T cells transformed to IL13Rα2-specific chimeric antigen receptor

Experimental method

200ul of a culture medium was put per well in 96 well tissue culture plate, and a target cell (1×10^5) was added. In order to measure cytotoxicity according to CAR expression rate, untransduced activated T cell and 10-70% of CAR-T cell specific to IL13Rα2 were put in the prepared 96 well tissue culture plate with the effector (1×10^5), and cultured at the same time

through the duplicate experiment. In addition, in order to verify whether the CAR-T cell shows number-dependent anticancer activity increase, serial dilution was performed from 7500 CAR-T and then put into the effector and cultured at the same time through the duplicate experiment. After 19 hours from the cultivation, the IFN-γ analysis experiment was carried out using the culture supernatant with ELISA analyzer (R&D systems) according to the guidelines of the analyzer manufacturer (Figures 7 and 8).

Experimental result

Generally, the activated T cell generates cytokine that is helpful in its growth and activation, and among them, IFN-γ is secreted by CD8 cell, CD4 T cell and NK cell, etc. and serves an important role in the inherent immune and adaptive immune responses. In particular, this serves an important role in moving T cell to the tumor site as well as inhibiting occurrence of cancer. Through the present experimental example, it was demonstrated whether the generation of IFN-γ is increased when the T cell expressing IL13Rα2-specific CAR met a target cell. According to the experimental method, the T cell expressing IL13Rα2-specific CAR was cultured at the same time as the target cell (HUVEC cell, U251 cell), and then IFN-γ was quantified through the ELISA analysis.

Figure 7 shows increased IFN-γ when binding to IL13Rα2 antigen according to the transduction ratio of the produced chimeric antigen receptor, and it can be understood that the

generation aspects of IFN-γ vary depending on the ratio of the CAR-T cell. In the case of using U251 which is the target cancer cell, the T cell in which the chimeric antigen receptor was untransduced rarely generated IFN-γ. In the case of the T cell in which the chimeric antigen receptor was transduced, the generation of IFN-γ increased by up to 30% ratio, and given that the generation was not increased anymore, it is determined that 30% ratio of the CAR-T cell is sufficient to kill the target cancer cell. Given that in the case of HUVEC which is a cell line that does not overexpress IL13Rα2, even if the ratio of CAR-T increases, the generation of IFN-γ does not increase, it seems that the generation of IFN-γ by CAR-T is specific to IL13Rα2 antigen. Fig. 8 shows increased IFN-γ according to the number of CAR+T cells in two donors, YY6 and YY7, as the number of cells increased, IFN-γ increased. This shows that in killing the cancer cell, the CAR-T cell is dependent on the number of cells.

Example 4: Evaluation of *in vivo* efficacy using YYB-103

In order to evaluate whether YYB-103 actually exhibits efficacy *in vivo*, a cancer cell was subcutaneously injected into a nude mouse to induce tumor, and after treating with YYB-103, the change of the tumor size and the persistence of CAR-T cell in the tumor tissue was confirmed.

Experimental Example 1: Production of a tumor nude mouse using U251 cell line and check for efficacy of YYB-103

The U251 cell line was subcutaneously injected into a nude mouse. After 9 days, control PBS and treatment group YYB-103 were intravenously administered once. Temozolomide (TMZ) and IL-2 were intraperitoneally and intravenously administered into the control group and the experimental group once a day for four days. The size of the tumors was measured after 12 days treatment, and after 15 days treatment, a postmortem was carried out for the measurement of the weight of the tumor tissue and the histologic analysis (Figure 9)

Experimental Result

As the result of measuring the size of the tumor after 12 days treatment to measure efficacy of YYB-103 which was administered into the established subcutaneous tumor nude mouse animal model, in the control group, the size of the tumor was reduced by about 44% from 234.8 mm3 to 132.4 mm3. However, in the case of administering YYB-103 as the treatment group, the size of the tumor was reduced by about 78% from 288.2 mm3 to 64.6 mm3. From this, it can be seen that the treatment group showed the therapeutic effect about 1.8 times as compared to the control group (Figure 9B). As the result of the measurement of the weight of tumor tissue, obtained by carrying out a postmortem after 15 days treatment, it can be seen that the weight of the tumor tissue of the nude mouse treated with the treatment group, YYB-103, was lighter than the weight of the tumor tissue of the mouse treated with the control group (Figure 9C). Together with this, when treated with YYB-103, it showed that

angiogenesis is inhibited in the tumor tissue, unlike the tumor tissue treated with the control group (Figure 11A and Figure 11B).

In order to verify that YYB-103 persists in the tumor tissue after 15 days treatment, staining was carried out using an anti-human CD3 antibody which is human T cell marker. As the result, it showed that the tumor tissue treated with the control group was not stained because the mouse was free of human T cell (Figure 10A).

It was confirmed that the tumor tissue treated with YYB-103, numerous human T cells existed, and this directly gave an influence on the reduction of the size of the tumor treated with YYB-103 (Figure 10B).

As the result of observing the tumor tissue by H&E staining on the tumor tissue after 15 days treatment, it is seen that in the group that was not treated with YYB-103, many blood vessels were observed. Thus, it seems that due to YYB-103, angiogenesis is inhibited in the tumor site, which results in less aggressive tumor (Figure 11A & Figure 11B).

Example 5: Construction of CAR capable of recognizing various solid tumors and angiogenic blood vessels, and production of PG13 cell line stably expressing CAR.

If angiogenesis which allows new blood vessel to grow in the tumor site is inhibited, the transition and growth of the cancer cell can be inhibited. Successful anti-cancer CAR therapies require not only antigen-specific CAR-T cells but also accessing CAR-T cells to cancer cells and maintaining CAR-T cell function in the immunosuppressive tumor microenvironment. Therefore, anti-angiogenic CAR was produced in order to achieve the object (Figure 12A).

CAR targeting EGFRvIII which is major tumor antigen of glioblastoma, lung cancer, etc. was produced (Figure 13A).

EphA2 (Membrane-bound erythropoietin-producing hepatocellular receptor tyrosine kinase class A2) is overexpressed in breast cancer, prostate cancer, bladder cancer, skin cancer, lung cancer, ovarian cancer, and brain cancer, etc., and thus CAR targeting EphA2 was produced (Figure 14A).

Integrin alpha(V) beta(3) (αVβ3), which is glycoprotein membrane receptor, is highly expressed in activated tumor epithelial cell. CAR targeting αVβ3 which is a resistant marker of carcinoma stemness and receptor tyrosine kinase inhibitors (RTKIs) such as erlotinib of pancreatic cancer, lung cancer and breast cancer was produced (Figure 15A).

GPC1 is important for efficient growth, transition and angiogenesis of cancer cell, and GPC1 is overexpressed in pancreatic cancer, breast cancer, glioblastoma (Figure 16A).

Experimental Example 1: Check for CAR expression rate on PG13 cell surface transformed to CAR capable of recognizing Angiogenic blood vessels, EGFRvIII, EphA2, Integrin αVβ3 or GPC1

Experimental method (flow cytometric analysis)

For flow cytometry (>30,000 events), BD LSRII equipment (Becton Dickinson) and BD FACSDiva software (Becton Dickinson) were used. Specifically, before adding an antibody, the cell was washed once with PBS containing 2% bovine serum albumin. After washing, the cell was reacted with the respective antibodies for 30 minutes at 4℃ in the state where light was blocked and then washed once, and checked the CAR expression rate on PG13 cell surface transformed to CAR, anti-human Fibronectin monoclonal antibody, PE-conjugated anti-human EphA2 monoclonal antibody (R&D Systems) PE-conjugated anti-human alpha v beta 3 monoclonal antibody (BioLegend) was used, and in the case of the antibody where fluorescence is not combined, PE conjugated anti-mouse IgG1 monoclonal antibody (Santa Cruz Biotechnology) or donkey anti-goat IgG phycoerythrin secondary antibody (R&D systems) were additionally used to conduct fluorescence staining.

Experimental result

As the result of confirming the expression rates of the respective CARs in the transduced PG13 cell line, it was shown that chimeric antigen receptor is expressed on the surface of almost of living PG13 cell lines (Figure 12B, Figure 13B, Figure 14B, Figure 15B and Figure 16B).

INDUSTRIAL APPLICABILITY

The present invention relates to CAR-T cells that have been rapidly developed in the cancer treatment field, and is applicable to the medical industry in the patient-specific cancer treatment field.

Sequence Listing Free Text

SEQ ID NO. 1 *{Sequence of antigen binding wild type IL-13 domain binding to IL13Rα2}*

Length: 112

Type: ligand protein

Name: human

Sequence:

GPVPPSTALRELIEELVNITQNQKAPLCNGSMVWSINLTAGMYCAALESLINVSGCSAI

EKTQRMLSGFCPHKVSA GQFSSLHVRDTKIEVAQFVKDLLLHLKKLFREGQFN

SEQ ID NO. 2 {antigen binding domain capable of binding to an antigen associated with an

angiogenic activity}

Length: 92

Type: ligand protein

Name: human

Sequence:

EVVAATPTSLLISWRHPHFPTRYYRITYGETGGNSPVQEFTVLQPPSTATISGLKPGVD

YTITVYAVVERNGRELNT PPISINYRTHHHHHH

SEQ ID NO. 3 *{antigen binding domain binding to EGFRvIII}*

Length: 252

Type: scFv protein

Name: human

Sequence:

QVQLQESGGGLVKPGGSLKLSCAASGFTFSKFGMSWVRQTPDKRLEWVASISTGGYN

TFYSDNVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYYCARGYSSTSFAMDYWGQG

TMVTVSSGSTSGSGKPGSGEGSDIQMTQSPSSLSASVGDRVTITCMTSTDIDDDMNWY

QQKPGKTPKLLIYEGNTLRPGVPSRFSGSGSGTDFIFTISSLQPEDIATYYCLQSFNV

PLTFGGGTKVEIKEQKLISEEDL

SEQ ID NO. 4 *{antigen binding domain binding to EphA2}*

Length: 141

Type: ligand protein

Name: human

Sequence:

DRHTVFWNSSNPKFRNEDYTIHVQLNDYVDIICPHYEDHSVADAAMEQYILYLVEHE

EYQLCQPQSKDQVRWQCN

RPSAKHGPEKLSEKFQRFTAFALAKEFKAGHSYYYISKPIHQHEDRCLRLKVTVSGEQ

KLISEEDL

SEQ ID NO. 5 *{antigen binding domain binding to αVβ3}*

Length: 104

Type: ligand protein

Name: human

Sequence:

VSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGETGGNSPVQEFTVPGSKSTATI

SGLKPGVDYTITVYAVTPRGDWNEGSKPISINYRTEQKLISEEDL

SEQ ID NO. 6 *{antigen binding domain binding to glypican1}*

Length: 418

Type: ligand protein

Name: human

Sequence:

TSPCDNFDCQNGAQCIVRINEPICQCLPGYQGEKCEKLVSVNFINKESYLQIPSAKVRP

QTNITLQIATDEDSGILLYKGDKDHIAVELYRGRVRASYDTGSHPASAIYSVETINDG

NFHIVELLALDQSLSLSVDGGNPKIITNLSKQSTLNFDSPLYVGGMPGKSNVASLRQAP

GQNGTSFHGCIRNLYINSELQDFQKVPMQTGILPGCEPCHKKVCAHGTCQPSSQAGFT

CECQEGWMGPLCDQRTNDPCLGNKCVHGTCLPINAFSYSCKCLEGHGGVLCDEEED

LFNPCQAIKCKHGKCRLSGLGQPYCECSSGYTGDSCDREISCRGERIRDYYQKQQGY

AACQTTKKVSRLECRGGCAGGQCCGPLRSKRRKYSFECTDGSSFVDEVEKVVKCGC

TRCVSEQKLISEEDL

SEQ ID NO. 7 *{Wild type CD28}*

Length: 41

Type: protein

Name: human

Sequence:

RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS

SEQ ID NO. 8 *{TNFRSF9}*

Length: 42

Type: protein

Name: human

Sequence:

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL

SEQ ID NO. 9 *{hinge region sequence -1}*

Length: 47

Type: protein

Name: human

Sequence:

KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD

SEQ ID NO. 10 *{hinge region sequence-2}*

Length: 45

Type: protein

Name: human

Sequence:

KPTTTPAPRPPTPAPTIASQPLSLRPEAARPAAGGAVHTRGLDFA

SEQ ID NO. 11 *{transmembrane domain sequence-1}*

Length: 21

Type: protein

Name: human

Sequence:

IYIWAPLAGTCGVLLLSLVIT

SEQ ID NO. 12 *{transmembrane domain sequence-2}*

Length: 23

Type: protein

Name: human

Sequence:

LAYLLDGILFIYGVILTALFLRV

SEQ ID NO. 13 *{CD3ζ comprising extra glutamine}*

Length: 113

Type: protein

Name: human

Sequence:

RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQ

EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPP

R

SEQ ID NO. 14 *{YYB 103}*

Length: 359

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLT

AGMYCAALESLINVSGCSAIEKTQDMLDGFCPHKVSAGQFSSLHVRDTKIEVAQFVK

DLLLHLKKLFKEGQFNGGGPRKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH

TRGLDFACDIYIWAPLAGTCGVLLLSLVITKRGRKKLLYIFKQPFMRPVQTTQEEDGC

SCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP

EMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT

KDTYDALHMQALPPR

SEQ ID NO. 15 *{YYB 103A}*

Length: 400

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLT

AGMYCAALESLINVSGCSAIEKTQDMLDGFCPHKVSAGQFSSLHVRDTKIEVAQFVK

DLLLHLKKLFKEGQFNGGGPRKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH

TRGLDFACDIYIWAPLAGTCGVLLLSLVITRSKRSRGGHSDYMNMTPRRPGPTRKHY

QPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRV

KFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEG

LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 16 *{YYB-103B, IL13(E11K.R107K).29.TNFRSF9.CD3ζ}*

Length: 400

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSGPVPPSTALRKLIEELVNITQNQKAPLCNGSMVWSINLT

AGMYCAALESLINVSGCSAIEKTQRMLSGFCPHKVSAGQFSSLHVRDTKIEVAQFVKD

LLLHLKKLFKEGQFNGGGPRKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHT

RGLDFACDIYIWAPLAGTCGVLLLSLVITRSKRSRGGHSDYMNMTPRRPGPTRKHYQP

YAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKF

SRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGL

YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 17 *{YYB 104}*

Length: 339

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSEVVAATPTSLLISWRHPHFPTRYYRITYGETGGNSPVQEF

TVLQPPSTATISGLKPGVDYTITVYAVVERNGRELNTPPISINYRTHHHHHHGGGPRKP

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVL

LLSLVITKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADA

PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQK

DKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 18 *{YYB 104-1}*

Length: 308

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSEVVAATPTSLLISWRHPHFPTRYYRITYGETGGNSPVQEF

TVLQPPSTATISGLKPGVDYTITVYAVVERNGRELNTPPISINYRTHHHHHHGGGPRKP

TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVL

LLSLVITRSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLY

IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELN

LGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGE

RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 19 *{YYB 105}*

Length: 497

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSQVQLQESGGGLVKPGGSLKLSCAASGFTFSKFGMSWVR

QTPDKRLEWVASISTGGYNTFYSDNVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYY

CARGYSSTSFAMDYWGQGTMVTVSSGSTSGSGKPGSGEGSDIQMTQSPSSLSASVGD

RVTITCMTSTDIDDDMNWYQQKPGKTPKLLIYEGNTLRPGVPSRFSGSGSGTDFIFTIS

SLQPEDIATYYCLQSFNVPLTFGGGTKVEIKEQKLISEEDLGGGPRKPTTTPAPRPPTPA

PTIASQPLSLRPEAARPAAGGAVHTRGLDFALAYLLDGILFIYGVILTALFLRVKRGRK

KLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELKFSRSADAPAYQQGQNQLYNE

LNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMK

GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 20 *{YYB 105-1}*

Length: 538

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSQVQLQESGGGLVKPGGSLKLSCAASGFTFSKFGMSWVR

QTPDKRLEWVASISTGGYNTFYSDNVKGRFTISRDNAKNTLYLQMSSLKSEDTAMYY

CARGYSSTSFAMDYWGQGTMVTVSSGSTSGSGKPGSGEGSDIQMTQSPSSLSASVGD

RVTITCMTSTDIDDDMNWYQQKPGKTPKLLIYEGNTLRPGVPSRFSGSGSGTDFIFTIS

SLQPEDIATYYCLQSFNVPLTFGGGTKVEIKEQKLISEEDLGGGPRKPTTTPAPRPPTPA

PTIASQPLSLRPEAARPAAGGAVHTRGLDFALAYLLDGILFIYGVILTALFLRVRSKRSR

GGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQT

TQEEDGCSCRFPEEEEGGCELKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR

RGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQG

LSTATKDTYDALHMQALPPR

SEQ ID NO. 21 *{YYB 106}*

Length: 388

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSDRHTVFWNSSNPKFRNEDYTIHVQLNDYVDIICPHYEDH

SVADAAMEQYILYLVEHEEYQLCQPQSKDQVRWQCNRPSAKHGPEKLSEKFQRFTAF

ALAKEFKAGHSYYYISKPIHQHEDRCLRLKVTVSGEQKLISEEDLGGGPRKPTTTPAPR

PPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVIT

KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQ

GQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAE

AYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ ALPPR

SEQ ID NO. 22 *{YYB 106-1}*

Length: 429

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSDRHTVFWNSSNPKFRNEDYTIHVQLNDYVDIICPHYEDH

SVADAAMEQYILYLVEHEEYQLCQPQSKDQVRWQCNRPSAKHGPEKLSEKFQRFTAF

ALAKEFKAGHSYYYISKPIHQHEDRCLRLKVTVSGEQKLISEEDLGGGPRKPTTTPAPR

PPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVIT

RSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFM

RPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREE

YDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG

HDGLYQGLSTATKDTYDALH MQALPPR

SEQ ID NO. 23 *{YYB 107}*

Length: 351

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSVSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGET

GGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTPRGDWNEGSKPISINYRTEQKL

ISEEDLGGGPRKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIY

IWAPLAGTCGVLLLSLVITKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC

ELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKN

PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA

LPPR

SEQ ID NO. 24 *{YYB 107-1}*

Length: 392

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSVSDVPRDLEVVAATPTSLLISWDAPAVTVRYYRITYGET

GGNSPVQEFTVPGSKSTATISGLKPGVDYTITVYAVTPRGDWNEGSKPISINYRTEQKL

ISEEDLGGGPRKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIY

IWAPLAGTCGVLLLSLVITRSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAA

YRSKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAY

QQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKM

AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 25 *{YYB 108}*

Length: 665

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSTSPCDNFDCQNGAQCIVRINEPICQCLPGYQGEKCEKLVS

VNFINKESYLQIPSAKVRPQTNITLQIATDEDSGILLYKGDKDHIAVELYRGRVRASYD

TGSHPASAIYSVETINDGNFHIVELLALDQSLSLSVDGGNPKIITNLSKQSTLNFDSPLY

VGGMPGKSNVASLRQAPGQNGTSFHGCIRNLYINSELQDFQKVPMQTGILPGCEPCH

KKVCAHGTCQPSSQAGFTCECQEGWMGPLCDQRTNDPCLGNKCVHGTCLPINAFSY

SCKCLEGHGGVLCDEEEDLFNPCQAIKCKHGKCRLSGLGQPYCECSSGYTGDSCDREI

SCRGERIRDYYQKQQGYAACQTTKKVSRLECRGGCAGGQCCGPLRSKRRKYSFECT

DGSSFVDEVEKVVKCGCTRCVSEQKLISEEDLGGGPRKPTTTPAPRPPTPAPTIASQPL

SLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITKRGRKKLLYIFK

QPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLG

RREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR

RGKGHDGLYQGLSTATKDTYDALHMQALPPR

SEQ ID NO. 26 *{YYB 108-1}*

Length: 706

Type: protein

Name: human

Sequence:

MGWSCIILFLVATATGVHSTSPCDNFDCQNGAQCIVRINEPICQCLPGYQGEKCEKLVS

VNFINKESYLQIPSAKVRPQTNITLQIATDEDSGILLYKGDKDHIAVELYRGRVRASYD

TGSHPASAIYSVETINDGNFHIVELLALDQSLSLSVDGGNPKIITNLSKQSTLNFDSPLY

VGGMPGKSNVASLRQAPGQNGTSFHGCIRNLYINSELQDFQKVPMQTGILPGCEPCH

KKVCAHGTCQPSSQAGFTCECQEGWMGPLCDQRTNDPCLGNKCVHGTCLPINAFSY

SCKCLEGHGGVLCDEEEDLFNPCQAIKCKHGKCRLSGLGQPYCECSSGYTGDSCDREI

SCRGERIRDYYQKQQGYAACQTTKKVSRLECRGGCAGGQCCGPLRSKRRKYSFECT

DGSSFVDEVEKVVKCGCTRCVSEQKLISEEDLGGGPRKPTTTPAPRPPTPAPTIASQPL

SLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITRSKRSRGGHSDY

MNMTPRRPGPTRKHYQPYAPPRDFAAYRSKRGRKKLLYIFKQPFMRPVQTTQEEDGC

SCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP

EMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT

KDTYDALHMQALPPR

What is claimed is:

1. A chimeric antigen receptor comprising an antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain, characterized in that the costimulatory domain is mutated CD28 or TNFRSF9.

2. The chimeric antigen receptor according to claim 1, characterized in that in the mutated CD28, the amino acid in the 6th to 9th positions of the amino acid sequence indicated with Sequence No. 7 is substituted from RLLH to RGGH or an amino acid having the similar property to RGGH.

3. The chimeric antigen receptor according to claim 2, characterized in that in the amino acid having the similar property to RGGH, glycine (G) is substituted with alanine(A), valine(V), leucine(L) or isoleucine(I).

4. The chimeric antigen receptor according to any one of claims 1 to 3, characterized in that the costimulatory domain consists of mutated CD28 and TNFRSF9.

5. A chimeric antigen receptor comprising an antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain,

characterized in that three glycine or an amino acid having the similar property to glycine are additionally introduced between the antigen binding domain and the hinge region.

6. The chimeric antigen receptor according to claim 5, characterized in that the amino acid having the similar property to glycine is alanine(A), valine(V), leucine(L) or isoleucine(I).

7. A chimeric antigen receptor comprising an antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain, characterized in that the antigen binding domain binds to an antigen selected from the group consisting of IL13Rα2, an antigen associated with an angiogenic activity, EGFRvIII, EphA2, αVβ3, and glypican1.

8 The chimeric antigen receptor according to claim 7, characterized in that the antigen binding domain binds to IL13Rα2.

9. The chimeric antigen receptor according to claim 8, characterized in that the antigen binding domain has the amino acid sequence where the 11th position of the amino acid sequence indicated with Sequence No. 1 is substituted with lysine and the 107th position is substituted lysine.

10. The chimeric antigen receptor according to claim 8, characterized in that in the antigen binding domain, the 11th, 64th, 67th, and 107th positions of the amino acid sequence indicated with Sequence No. 1 are substituted with lysine(K), aspartic acid(D), aspartic acid(D) and lysine(K), or an amino acid similar thereto.

11. The chimeric antigen receptor according to claim 10, characterized in that the amino acid substituted with an amino acid similar thereto is substituted with arginine (R) or histidine (H), instead of lysine (K), in the 11th position; with glutamic acid(E), instead of aspartic acid(D), in the 64th and 67th positions; and histidine (H), instead of lysine (K), in the 107th position.

12. A chimeric antigen receptor comprising an antigen binding domain; a hinge region; a transmembrane domain; a costimulatory domain; and a cytoplasmic signaling domain, characterized in that the cytoplasmic signaling domain uses a CD3ζ signaling domain of a normal person where extra Glutamine is comprised.

13. The chimeric antigen receptor according to claim 12, characterized in that the extra Glutamine is glutamine present in the 50th position of the CD3ζ signaling domain of a normal person.

14. The chimeric antigen receptor according to claim 13, characterized in that the CD3ζ

signaling domain is sequence indicated with Sequence No. 13.

15. The chimeric antigen receptor according to claim 1, indicated with Sequence No. 14.

16. The chimeric antigen receptor according to claim 1, indicated with Sequence No. 15.

17. CAR-T cell in which the chimeric antigen receptor according to any one of claim 1, 5, 7, 10, 12 or 16 is expressed.

18. The chimeric antigen receptor according to claim 7, binding to an antigen associated with an angiogenic activity indicated with Sequence No. 17 or Sequence No. 18.

19. The chimeric antigen receptor according to claim 7, binding to EGFRvIII indicated with Sequence No. 19 or Sequence No. 20.

20. The chimeric antigen receptor according to claim 7, binding to EphA2 indicated with Sequence No. 21 or Sequence No. 22.

21. The chimeric antigen receptor according to claim 7, binding to αVβ3 indicated with Sequence No. 23 or Sequence No. 24.

22. The chimeric antigen receptor according to claim 7, binding to glypican1 indicated with Sequence No. 25 or Sequence No. 26.

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