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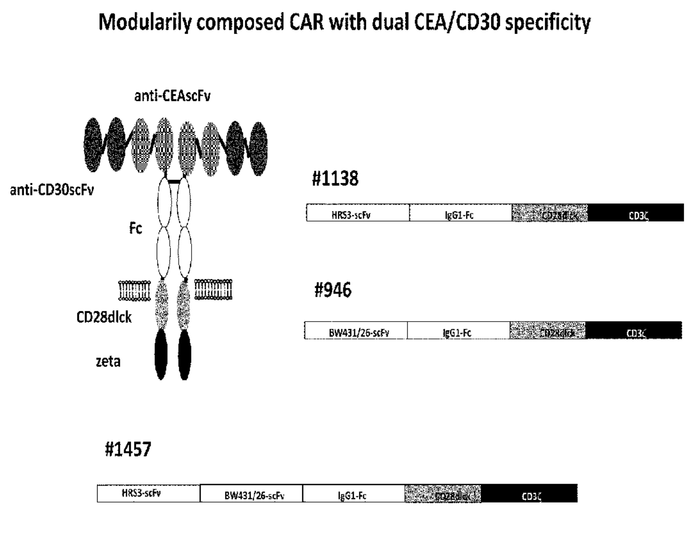

Figure 1

(57) Abstract: In a first aspect, the present invention relates to a recombinant polypeptide containing a domain comprising at least two antibody units whereby the first antibody unit is an anti-CD30 single chain antibody unit while the second antibody unit is a antibody unit being specific for an antigen present on the surface of a predetermined target cell. In particular, the present invention relates to a recombinant polypeptide containing at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologs thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain. In a further aspect, the present invention relates to a nucleic acid molecule encoding the polypeptide according to the present invention, as well as vectors and cells containing the same. Moreover, lymphocytes are provided, in particular T-cells like $CD8^+$ or a $CD4^+$ T-cell expressing on its surface chimeric antigen receptors containing an anti-CD30 single chain antibody unit and an antibody unit whereby said antibody unit being specific for an antigen present on the surface of a predetermined target cell. Immune cells modified with thepolypeptide show improved functions, in particular in the treatment of cancer, in particular $CD30^-$ cancer. That is, the cells are for use in adapted cell therapy for treating cancer in a subject in need thereof.

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Chimeric antigen receptor and its use

In a first aspect, the present invention relates to a recombinant polypeptide containing a domain comprising at least two antibody units whereby one antibody unit is an anti-CD30 single chain antibody unit while another antibody unit is an antibody unit being specific for an antigen present on the surface of a predetermined target cell. In particular, the present invention relates to a recombinant polypeptide containing at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologous thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain. The recombinant polypeptide can likewise be arranged in the order where the domain comprising at least two antibody units has the order: a first domain containing an antibody unit specific for a tumor-associated antigen and the second domain containing an anti-CD30 single chain antibody unit. In a further aspect, the present invention relates to a nucleic acid molecule encoding the polypeptide according to the present invention, as well as vectors and cells containing the same. Moreover, lymphocytes are provided, in particular T-cells like $CD8^+$ or a $CD4^+$ T-cell, expressing on its surface chimeric antigen receptors containing an anti-CD30 single chain antibody unit and an antibody unit whereby said antibody unit being specific for an antigen present on the surface of a predetermined target cell.

These cells are particularly useful in treating cancer, in particular $CD30^-$ cancer. That is, the cells are for use in adoptive cell therapy for treating cancer in a subject in need thereof.

Prior art

Adoptive T-cell transfer has shown significant efficacy in the treatment of malignancies and can be curative in patients with various diseases including leukaemia or Epstein Barr virus associated malignancies. Usually, patient derived T-cells are engineered *ex vivo* to express a recombinant T-cell receptor or, alternatively, a Chimeric Antigen Receptor. Said Chimeric Antigen Receptor (CAR) is typically composed of an extracellular antigen binding domain derived from an antibody and an intracellular T-cell activation domain derived from the T-cell receptor endodomain. In contrast to the physiological TCR, the CAR is composed of one single polypeptide chain that combines antigen binding via the extracellular moiety with a T-cell activation machinery provided by the intracellular signalling moiety. Thus, due to the antibody derived binding domain, CAR modified T-cells recognize their target, usually, a cell surface antigen, independently of the Major Histocompatibility Complex (MHC) presentation of antigen and are not compromised by tumour cell variants with lowered or deficient antigen possessing which represents a commonly observed mechanism of tumour immune escape. CARs are in the focus of research in the recent years. In particular, their capacity to target and lyse pre-defined cells are in the focus of immunotherapy. Recent clinical trials have underscored the potential of adaptive therapy of cancer with CAR-redirected T-cells. For example, neuroblastoma patients treated with GD2 Ganglioside-specific CAR T-cells showed some encouraging anti-tumour effects although the T-cells persist only for a brief period. Further studies proved the concept that CAR engineered T-cells can initiate a productive anti-tumour response in patients suffering from various malignancies. The CAR approach differs to other antibody mediated immune therapy strategies, e.g. by using immunotoxins, in so far that engineered cells are used instead of single molecules.

In recent years, efforts have been done in the optimization of the CAR design, see e.g. Bridgeman J.S., et al., Curr Gene Ther 2010, 10, 77-90. However, many challenges remain, in particular, the necessity of a more effected anti-tumour response and prolonging T-cell survival allowing long term T-cell persistence of said engineered T-cells in the body. In addition, the co-stimulatory signals required for a successful clinical application remains to be illusive, e.g. see Hombach A, et al., Curr Mol Med. 2013 Aug;13(7):1079-88. Hence, there is ongoing work on optimizing CAR for various approaches including adaptive immunotherapy.

Hombach A., et al., Gene Therapy, 2010, 17, 1206-1213 describe the modification of the IgG1 Fc spacer domain in the extracellular moiety of Chimeric Antigen Receptors for avoiding off-target activation and unintended initiation of an innate immune response. Therein, T-cells engineered with modified CAR, either an anti-CD30 CAR or an anti-CEA CAR, have been used. As demonstrated therein, anti-CEA CAR T-cells are not effective against CEA negative cells and anti-CD30 CAR T-cells not against $CD30^-$ cells. The modification disclosed in this publication relates to the avoidance of target activation by unintended initiation of the immune response, e.g. due to cross reactivity with a spacer domain in the extracellular moiety of the CAR.

In Kofler et al., 2011, Mol. Ther. 19, 760-767 a CAR molecule is described having a CD28 endodomain combined with a CD3 endodomain and an antibody derived scFv ectodomain specific for CEA. It is described therein, that a deletion of the lck binding moiety in the CD28 CAR endodomain improves redirected anti-tumour activity in presence of T-regulatory cells without impairment of interferon-gamma secretion, proliferation and cytolysis. It is speculated, that the CAR with the modified CD28 endodomain expedite the implementation of adoptive T-cell therapy in patients with a variety of cancer types that are heavily infiltrated by regulatory T-cells (Treg cells).

A summary of the present knowledge on chimeric antigen receptor redirected T-cells is given in the review article of Chmielewski M., et al, frontiers in immunology, 2013, 4, 1-7 as well as in Melenhorst JJ, Levine BL., Cytotherapy. 2013 Sep;15(9):1046-53. In addition, a summary of adoptive therapy of cancer with CAR redirected T-cells is provided in Hombach A., et al., Curr Mol Med, 2013, 13(1), 1-10. Therein, the effects of CAR are summarized including co-stimulation activity as well as improvement and prolongation of the redirected anti-tumour T-cell response. In addition, the adverse effects of this kind of adaptive therapy are described including “cytokine storm” and “T-cell repression”.

Recently, Chmielewski et al, Immunol Reviews vol. 257, 2014, p83-90 discusses the possibility of CAR T-cells engineered with an inducible cytokine to modulate the tumor stroma.

Wilkie, S. et al., J. Clin. Immunol., 2012, 32, 1059-1070 describe a dual targeting of ErB2 and MUC1 in breast cancer using two separate CARs, each capable for full T cell activation. Kloss et al., Nat Biotechnol. 2013 Jan;31(1):71-5, report T cells engineered with two separate CARs which provide complementary signalling. The vectors described therein encode two separate CAR molecules expressed simultaneously by the same T cells.

Grada, Z. et al., Molecular Therapy-Nucleic Acids, 2013, vol. 2, page 105 relate to TanCAR, a novel bispecific CAR for cancer immunotherapy. The CAR molecules described therein are designed to identify ligands both being present on the target cell.

That is, currently used chimeric antigen receptor (CAR) modified T-cells for antigen-redirected targeting towards tumor cells show insufficient performance in the anti-tumor attack due to various reasons, in particular due to less amplification and cytolytic activity after adoptive transfer into the patient. Hence, a major obstacle of the strategy for clinical use is the insufficient performance of engineered T-cells in the long-term.

Further, beside beneficial effect of the CAR expressing T-cell in adoptive therapy, ample effects are known which presently hinder favourite development of respective therapy as mentioned above.

Brief description of the present invention

The present invention aims at improving persistence and performance of CAR T-cells.

In a first aspect, the present invention relates to a recombinant polypeptide containing at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologs thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain. In particular, the present invention relates to a recombinant peptide wherein the first domain comprises the anti-CD30 single chain antibody unit in combination with an antibody unit whereby this antibody unit is specific for a tumor-associated antigen. In a favourable embodiment, the anti-CD30 single chain antibody unit is the HRS3 scFv of SEQ ID No. 2 while the antibody unit is a unit binding to the tumor-associated antigen of carcinoembryonic antigen (CEA), in particular of SEQ ID No. 4. In a further aspect, the present invention relates to a nucleic acid molecule encoding the polypeptide according to the present invention as well as a vector comprising said nucleic acid sequence. Moreover, a cell, cell line or host cell containing said nucleic acid sequence or said vector is provided.

In addition, the present invention is directed to a lymphocyte, in particular, a T-cell, like a $CD8^{+}$ and/or a $CD4^{+}$ T-cell, expressing on its surface chimeric antigen receptors having in their extracellular domain two antibody units, an antibody unit being specific for an antigen present on the surface of a predetermined target cell, and an anti-CD30 single chain antibody unit. These units are either present in a single polypeptide as defined herein or present in different functional chimeric antigen receptors. These cells are particularly useful for treating tumor cells expressing the antigen on its surface but may not express CD30 on its surface. That is, by combining these two units, namely, the anti-CD30 single chain antibody unit and an antibody domain being specific for a tumor-associated antigen, tumor cells expressing the tumor specific antigen on its surface but being $CD30^{-}$ can be treated more efficiently. Hence, the present invention provides in a further aspect a method for treating subjects being afflicted with cancer whereby the tumor cells of said cancer express the antigen but are $CD30^-$(CD30 negative).

In particular, the cells useful for adoptive cell therapy according to present invention express on its surface chimeric antigen receptors having in the extracellular domain the antibody unit being specific for an antigen present on the surface of a predetermined cell, in particular, wherein the antibody unit is an antibody unit being specific for a tumor-associated antigen, and an anti-CD30 single chain antibody unit whereby these two units may be present either in a single polypeptide representing a CAR or may be present in different functional chimeric antigen receptors.

Brief description of the drawings

Figure 1: Schematic representation of the modular composition of the CAR molecules. #1138 represents the anti-CD30 CAR; #946 represents the anti-CEA CAR. The CAR #1457 is the bispecific CAR molecule according to the present invention composed of the anti-CD30 single chain antibody scFv HRS3, the anti-CEA scFv BW431/26 , the spacer domain IgG1-Fc, the trans-membrane domain of CD28 and the cytoplasmic signalling domain of the CD28ΔLCK and CD3zeta chain.

Figure 2: Peripheral blood lymphocytes were engineered with CARs by standard procedures. After cultivation $CD3^+$ cells where analysed for CAR expression utilising FITC-conjugated anti-CD3 and PE-conjugated anti-human IgG1Fc antibodies. As demonstrated, transduction was successfully conducted and T-cells express the anti-CD30/CEA CAR on the cell surface in similar amounts as T-cells transduced with the anti-CD30 and anti-CEA CAR, respectively.

Figure 3: Human T-cells were engineered with CARs as indicated with an initial CAR expression rate of anti-CEA-CAR: 22.2%; anti-CD30-CAR: 28.9%; anti-CEA/CD30-CAR: 22.8%. CAR T-cells were cultivated 300 hours in the presence of IL-2 and CAR expressing cells were monitored. CAR T-cells with a CD30 binding domain expand more efficiently than T-cell with the anti-CEA CAR only.

Figure 4: Engineered human T-cells expressing different kinds of CARs as indicated (initial CAR expression: anti-CEA-CAR: 8.5%; anti-CD30-CAR: 9.3 9%; anti-CEA/CD30-CAR: 8.2%). CAR T-cells (0.625-5 x 10^4 cells/well) where co-incubated for 24 hours with tumor cells (2.5-5x10^4 cells/well) as indicated. Three different types of cell lines where used being either positive for CEA or CD30 or as control negative for both CEA and CD30. The cell line being negative for both CEA and CD30 was not lysed while specific lysis of LS174T cells was observed which is a $CEA^+/CD30^-$ cell line. Therein, the anti-CEA/CD30 CAR T-cells demonstrate a higher specific lysis compared to the anti-CEA CAR T-cells.

Figure 5: The release of interferon-gamma, a marker for T-cell activation, during co-cultivation of different numbers of effector cells with the tumor cells is shown. After 24 hours co-cultivation the supernatants were collected and analysed for interferon-gamma by ELISA. The assay was performed in triplicate and the SD was determined. As shown, anti-CEA/CD30 CAR T-cells produced IFN-gamma when co-incubated with the $CEA^+/CD30^-$ LS174T-cells as well as with the CEA^- $CD30^+$ cells. No IFN-gamma was produced upon co-incubation with CEA^- $CD30^-$ Colo320 cells. T-cells with the anti-CEA CAR or anti-CD30 CAR released IFN-gamma only upon co-incubation with CEA^+ or $CD30^+$ cells, respectively.

Figure 6: Figure 6 shows the cytolytic activity of CAR CIK T-cells (cytokine induced killer cells with NKT-cell phenotype). It is demonstrated that enhanced killing of CEA^+ $CD30^-$ target cells by using the anti-CEA/CD30 CAR CIK T-cells is achieved. The tumor cell lines are the same as described in figure 2.

Figure 7: Figure 7 demonstrates improved in vivo killing of CEA^+ tumor cells by anti-CEA/CD30 CAR T-cells compared to the mono-specific anti-CEA CAR T-cells. Subtherapeutic doses of T-cells (2.5 x10^5 cells/mouse) without CAR and T-cells engineered either with the anti-CEA/CD30 CAR or the anti-CEA CAR were co-injected with CEA^+ $CD30^-$ C15A3 tumor cells (1x10^6 cells/mouse) into immune deficient Rag $^{-/-}$ common gamma chain $^{-/-}$ mice. For comparison, C15A3 tumor cells were injected without T-cells (w/o). Tumor volumes and area under curve (AUC) were determined. Significance was determined by Student´s T-test. Significant differences ($p<0.05$) were indicated by asterisks.

Figure 8: Figure 8A shows schematic representation of anti-CD30 CARs and the CD25/CEA bispecific CAR of Seq. ID. No. 18 encoded by the sequence of Seq. ID. No.17. The HRS3-scFv of CAR #1457 was exchanged by the anti-CD25-scFv RFT5. The backbone of the resulting #1576 CAR is identical to CAR # 1457 shown in Figure 1.

Figure 8B shows the specificity of cytotoxicity. CAR T cells (0.625-5 x 10^4 cells/well) where co-incubated for 24 hours with CEA+ or CEA- tumor cells (2.5-5x10^4 cells/well) as indicated. Only CAR T cells expressing the bispecific anti-CD30/CEA CAR lysed CEA+ target cells whereas CAR T cells with the bispecific anti-CD25/CEA CAR and CAR T cells with a monospecific anti CEA CAR lysed CEA+ targets with lower efficiency. CEA- tumor cells were not lysed demonstrating the CEA-specificity of CAR T cells.

Figure 9: Figure 9A is a schematic representation of the molecular composition of the CAR molecules with specificity for CD30/MUC1of Seq. ID. No. 20 encoded by the sequence of Seq. ID. No. 19 and CD30/TAG-72 of Seq. ID. No. 22 encoded by the sequence of Seq. ID. No. 21. B72.3 is used for the anti-TAG-72 CAR. Except for the anti-CEA scFv, the molecules are identical with the CAR shown in figure 1. Cells surface expression of CD30/Muc1 and CD30/TAG-72 CARs is demonstrated in Fig. 9B by flow cytometry.

Detailed description of the present invention

The inventors aim to provide new recombinant polypeptides containing at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologous thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain. These recombinant polypeptides represent bispecific CAR peptides. In an embodiment of the present invention, these CAR polypeptides are expressed by genetically engineered T-cells containing and eventually expressing these CAR polypeptides.

That is, in a further aspect, the present invention relates to a recombinant polypeptide being at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologous thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain.

The present invention likewise relates to the following domains, a first domain an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, and a second domain containing an anti-CD30 single chain antibody unit, optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain.

As used herein, the term “comprise” or “comprising” as well as the terms “contain” or “containing” includes the embodiment of “consist” or “consisting”.

The term “homolog” as used herein refers to molecules, either DNA or polypeptides, having a sequence homology of a certain amount, namely of at least 70%, like at least 80%, 90%, 95%, 96%, 97%, 98%, 99% of the nucleic acid sequence or the amino acid sequence it is referred to. Homology refers to the magnitude of identity between two sequences. Homolog sequences have the same or similar characteristics, in particular, have the same or similar property of the sequence as identified. For example, the homolog of the HRS3 scFv sequence of Seq. ID. No. 2 has the same or similar binding specificity to the CD30 molecule as it is the case for the HRS3 scFv molecule. Further, homologs include nucleic acid molecules encoding the same peptide but may vary in its sequence due to the degeneracy of the genetic code. Further, “identify” refers to the presence of identical amino acid or nucleic acid molecules in the order as described for the sequence it refers to. That is, in case of at least 90% identity, 90% or more of the nucleic acid and amino acid molecules, respectively, are identical at the respective positions. Unless otherwise identified, the terms “homology” and “identity” are used herein interchangeably. In particular, the homolog of the HRS3 scFv sequence of Seq. ID. No.2 include anti CD30 single chain antibody units binding to the same epitope recognized by the HRS3 scFv.

In addition, the term “genetically engineered” refers to cells being manipulated by genetic engineering. That is, the cells contain a heterologous sequence which does not naturally occur in said cells. Typically, the heterologous sequence is introduced via a vector system or other means for introducing nucleic acid molecules into cells including liposomes. The heterologous nucleic acid molecule may be integrated into the genome of said cells or may be present extra-chromosomally, e.g. in the form of plasmids. The term also includes embodiments of introducing genetically engineered, isolated CAR polypeptides into the cell.

Generally, CARs are fusion proteins, consisting of an extracellular antibody type recognition domain fused to intracellular T-cell signalling proteins. Typically, the ectodomain containing the antigen recognition region comprises a signal peptide and an antigen recognition unit. According to the present invention, the ectodomain comprises an anti CD30 single chain unit in combination with an antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen. It is preferred, that said single chain unit is a single chain unit selected from HRS3 scFv of SEQ. ID. No. 2 or homologous thereof having at least 70 % identity with SEQ ID No. 2 and binding specifically to CD30. Further, the single chain unit may be derived from other anti-CD30 antibodies like HRS4 or Ki-4. Said antibodies have the same binding specificity to the same epitope of CD30 as it is the case for the HRS3 antibody. That is, in an embodiment of the present invention, it is preferred that the anti-CD30 single chain unit is a single chain unit being specific to the epitope of the CD30 molecule recognized by the anti CD30 single chain unit HRS3 scFv of Seq. ID. No.2.

The ectodomain may be spaced apart from the transmembrane domain by the presence of a spacer domain. Said optional spacer domain links the antigen-binding domain to the transmembrane domain and it is preferred that said transmembrane domain is flexible enough to allow the antigen binding domain to orient in different directions to facilitate antigen recognition.

The transmembrane domain is typically a hydrophobic alpha helix that spans the membrane. Finally, the endodomain represents the signalling domain in the cytoplasma of the cells.

Further, the term “antibody unit being specific for an antigen present on the surface of a predetermined target cell” refers to the binding site derived from an antibody of any species either in form of a single polypeptide chain or multiple covalently or non-covalently associated polypeptide chains. These domains are characterized by specific binding to a molecule on the surface of a target cell. Any other binding moiety than an antibody can alternatively be used as targeting domain in a CAR, e.g., the binding moiety of a receptor like a cytokine receptor.

Moreover, the term “antibody unit being specific for a tumor-associated antigen” refers to a binding domain with specificity to a target molecule present on the cell surface expressed by tumor cells and composed of a polypeptide, carbohydrate or lipid or combinations thereof that are exclusively or preferentially expressed by malignant cells.

The term “functional CAR” refers to a CAR expressed by immune cells that by specific binding to the cognate target molecule activates the same immune cell to increase protein biosynthesis, cytokine secretion, cell proliferation and target cell lysis.

Moreover, the term “$CD30^+$ cells” or “$CD30^+$ cancer cells” refers to cells (either non-cancer or cancer cells) expressing on their surface the CD30 molecule.

The terms “non-tumor cells” and “tumor cells” as well as “non-cancer cells” and “cancer cells” are used herein interchangeably unless otherwise defined.

The term “CIK T-cells” or “NK T-cells” which are used herein interchangeably refers to a heterogeneous group of T-cells that share properties of both T-cells and natural killer (NK) cells. CAR CIK T-cells are obtained by known cultivation techniques to obtain firstly NK T-cells or CIK T-cells and, thereafter, engineering the same cells with the CAR by known methods.

The recombinant polypeptide according to the present invention represents a bispecific CAR also identified herein as dual specific CAR. In an embodiment, the order in the first domain is from the N-terminus to the C-terminus: i) an anti-CD30 single chain antibody unit and an antibody unit, said antibody unit being specific for an antigen present on the surface of a predetermined target cell, or ii) an antibody unit whereby said antibody unit being specific for an antigen present on the surface of a predetermined target cell, and an anti-CD30 single chain antibody unit.

It is preferred that the antibody unit being specific for an antigen present on the surface of a predetermined target cell is an antibody unit being specific for a tumor-associated antigen.

In an embodiment of the present invention, the CAR present in the T-cell comprises a leader sequence being located N-terminally to the first domain containing the anti CD30 single chain unit and the antibody unit.

In addition, in another embodiment, the anti-CD30 single chain antibody unit is a HRS3 scFv peptide, in particular, of SEQ. ID. No. 2, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 1. It has been recognized herein that an anti-CD30 single chain antibody fragment of the variable region (scFv), in particular, of HRS3, allows displaying the desired activity. In an embodiment, the anti CD30 single chain antibody fragment is a fragment recognizing and binding to the epitope of HRS scFv.

In another embodiment, the spacer domain of the CAR molecule is an IgG_1 hinge-CH2CH3 domain of SEQ. ID. No. 6 or homologs thereof having at least 70% identity therewith, preferably, the spacer domain is a mutated IgG_1 hinge-CH2CH3 domain according to SEQ. ID. No.6, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 5.

In some embodiments, a linker may be located between the spacer domain and the transmembrane domain.

Further, another embodiment relates to a T-cell with a chimeric antigen receptor wherein the transmembrane domain is derived from the CD28 molecule, e.g. the transmembrane domain of the CD28 molecule lacking the lck domain of SEQ. ID. No. 8, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 7.

The signalling domain or endodomain or intracellular domain which are used herein interchangeably, contains a CD3 zeta or FcEpsilon-Receptor gamma chain signalling chain or a co-stimulatory domain or both the CD3zeta and a co-stimulatory domain or the FcEpsilon-Rezeptor gamma chain and a co-stimulatory domain. For example, the intracellular domain is a CD3 zeta signalling domain of SEQ. ID. No. 10, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 9, or a homolog thereof having at least 70% homology. In another embodiment, the intracellular domain is the IgE Fc-Receptor gamma signalling domain of SEQ. ID. No. 12, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 11, or a homolog thereof having at least 70% identity. The signalling domain is responsible for the activation of the T-cells, in particular the cytotoxic activity and cytokine release including Interferon gamma secretion.

The CAR molecule may be a so-called second generation CAR molecule. Second generation CAR molecules have improved signalling domains additionally containing a second signalling (costimulatory) domain, e.g. derived from CD28, CD134 (OX40) or CD137 (4-1BB). Third generation CAR molecules contain a combined co-stimulatory signalling domain, e.g., CD28 combined with CD137 or CD134.

An overview about the CAR molecules is provided e.g. in Gilham D.E. et al., Trans. and Molecular Medicine, 2012, 18(7), 377-384.

In a preferred embodiment of the present invention, the T-cell is a T-cell with a chimeric antigen receptor wherein the chimeric antigen receptor is a polypeptide of SEQ. ID. No. 14, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 13. Said CAR is also referred to herein as #1457.

The anti-CD30 anti-CEA CAR #1457 is expressed on the surface of T-cells and is composed in the extracellular part of the anti-CD30 single chain fragment of variable region (scFv) antibody HRS3, the BW431/26 scFv antibody corresponding to Seq. ID. No. 4, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 3, corresponding to the anti-CEA antibody BW431/26 and the modified human IgG1 CH2CH3 domain as spacer between scFv and the trans-membrane domain. The modification of the IgG1 domain consists of point mutations to convert the wild-type amino acid sequence PELLGGP X_{13} MISRT (Seq. ID. No. 15) to PPVA-

GP X_{13} MIART (Seq. ID. No. 16) which reduces unintended binding of the CAR Fc domain to Fc receptors on other cells like innate immune cells which would mediate their activation. The transmembrane and intracellular membrane proximal part of CAR # 1457 is derived from human CD28 and is fused to the intracellular part of human CD3zeta. The CD28 sequence is mutated at P560 > A560, P563 > A563, P564 > A564 (Kofler et al., Mol. Ther. 19, 760 - 767 (2011). Thereby the CD28 binding site for the lck kinase is destroyed with the consequence that activation of the lck signalling pathway and subsequent CAR mediated IL-2 secretion is prevented. Pre-clinical models imply that Treg cell mediated repression of CAR T-cell effector functions is reduced under these conditions.

As an example, the recombinant polypeptide is the polypeptide of SEQ ID No. 14, e.g. encoded by the nucleic acid sequence of Seq. ID. No. 13 correspond to the #1457 CAR shown in figure 1.

In another embodiment, the recombinant polypeptide is a polypeptide wherein the antibody unit binds to a tumor-associated antigen including any one of carcinoembryonic antigen (CEA), CA19-9, CA72-4 also known as TAG-72, PSCA, Muc-1, HMW-MAA, p97 melanotransferrin, fetal actelycholin receptor, ErbB2 (Her2/neu), multi-drug-resistance protein (MDR), CD19, CD20, TOSO.

For example, the CAR #1457 of Seq. ID. No. 14 correspond to a polypeptide wherein the antibody unit being specific to CEA. Further, CARs useful according to the present invention are shown in figure 9, namely, CD30/MUC-1 CAR or CD30/Tag72 CAR. These CARs are #1587, anti-CD30/MUC1, of Seq. ID. No. 20 encoded by the sequence of Seq. ID. No. 19, and #1650 anti-CD30/TAG-72 of Seq. ID. No. 22 encoded by the sequence of Seq. ID. No. 21.

In another aspect, the antibody unit binds to viral antigens, in particular, hepatitis virus B-associated antigen S or L, cytomegalovirus-associated antigen or other viral antigens described in the art as being useful for detecting and, eventually, destroying virus infected cells.

As demonstrated in the examples, T-cells (either CD4 or CD8 T-cells) or CIK-cells demonstrate specific lysis of cells not expressing CD30 but expressing the antigen to which the antibody unit is specifically binding only. That is, surprisingly the percentage of specific lysis is higher with the bispecific CAR molecule compared to the monospecific molecule containing the antibody binding unit specific for the antigen present on the surface of the predetermined target cell only.

It is preferred, that the target cell is a tumor cell. Further, the target cell may be a virus loaded cell, e.g. a cell infected with a virus.

Moreover, the predetermined target cell may be a cell involved in autoimmune defects in a subject. In that particular case suppressor cells, in particular regulatory T-cells, are modified with the described CAR in order to repress the acute inflammatory immune reaction in the targeted tissue. The CAR binds to tissue antigen with one scFv, e.g., anti-HLA B27, and to CD30 with the other scFv.

Although not expressed by the predetermined target cells, the anti-CD30 single chain antibody unit present in the CAR molecule increases specific lysis of the target cells. Furthermore, it has been surprisingly shown that in case of CD30 negative cells the percentage of specific lysis can be increased as demonstrated in the examples. This is not only true for lysis by CAR expressing T-cells but also by CAR CIK-cells. It is considered that autostimulation of the CAR T-cells allows to increase specific lysis of the target cells.

In addition, the present invention provides nucleic acid molecules comprising the nucleic acid sequence encoding the polypeptide according to the present invention. Furthermore, vectors are provided comprising the nucleic acid sequence according to the present invention encoding the polypeptide as described. The skilled person is well aware of suitable vector systems and vectors, in particular, vectors allowing transfection and transduction of eukaryotic cells, in particular, T-cells.

Moreover, the present invention provides a cell, cell line or a host cell containing the vector according to the present invention or a nucleic acid molecule according to the present invention. Preferably, said cell, cell line or host cell is a T-cell, e.g., a $CD4^+$ T-cell or a $CD8^+$ T-cell.

Further, the present invention provides a kit or system containing the vector according to the present invention, the cell, cell line or host cell according to the present invention, or the polypeptide according to the present invention or a nucleic acid molecule according to the present invention or mixtures thereof for use in the production of T-cells expressing the chimeric antigen receptor. The kit or system according to the present invention may contain further components including means for introducing the vector, polypeptide or nucleic acid molecules into the cells. The skilled person is well aware of suitable means for doing so.

Moreover, the present invention provides lymphocytes, in particular, T-cells, like CD8 and/or CD4 T-cells expressing on its surface chimeric antigen receptors having in the extracellular domain an antibody unit, said antibody unit being specific for an antigen present on the surface for an antigen present on the surface of a predetermined target cell, and an anti-CD30 single chain antibody unit expressed by said cells. The units may be present either in a single polypeptide, e.g. a polypeptide as defined herein, or, alternatively, may be present in different chimeric antigen receptors.

In another aspect, the lymphocyte may be a CAR CIK-T-cell as defined herein.

The lymphocytes including the $CD8^+$, $CD4^+$ or CIK-T-cells demonstrate higher specific lysis of the target cells, thus, reducing side effects and, in addition, overcoming the problem of insufficient performance in the anti-tumor attach due to various reasons, for example, less amplification and cytolytic activity after adopted transfer into the patient.

It is considered that the presence of the anti-CD30 single chain antibody unit allows to amplify the genetically engineered lymphocytes and improve persistence of the same, thus, overcoming the problem of insufficient performance.

The cells are particularly useful in treating cancer, in particular $CD30^-$ cancer whereby said cancer is a cancer expressing on the cancer cells the antigen to which the anti-tumor antigen antibody is directed to.

In a further aspect, the present invention relates to the use of immune cells, e.g. the T-cell with a chimeric antigen receptor according to the present invention in adaptive cell therapy for treating $CD30^-$ or $CD30^+$ cancer in a subject in need thereof. For instance the $CD30^+$ cancer may be Hodgkin's lymphoma, anaplastic large cell lymphoma, acute lymphocytic leukaemia, cutaneous lymphoma, mycosis fungoides, lymphoproliferative diseases, systemic mastocytosis, teratocarcinoma, stem cell derived malignancies, or cancer stem cells or others. In particular the invention relates to the use of such immune cells for the treatment of CD30⁻ cancers, e.g., breast, lung, prostate, pancreas, gastrointestinal carcinomas, neuronal cancer and others. Thus, the present invention may foster adaptive immune therapy by CAR T-cells.

The present invention is further described by way of examples. Said examples illustrate the invention further without limiting the same thereto.

Examples

Preparation of CAR #1457

The expression cassette of the bispecific anti-CD30/CEA-CAR was generated as follows: The anti-CD30-scFv (HRS3) and the anti-CEA-scFv (BW431/26) were PCR amplified and flanked with a linker by overlapping sequences at the 3´terminus of the CD30-scFv and the 5´terminus of the anti-CEA-scFv while the linker coding for a 40 AA (Gly(4)-Ser(1))5 linker. DNAs of scFvs were assembled by SOE PCR, digested with NcoI and BamHI and ligated into the NcoI/BamHI site of the retroviral vector pBullet-HRS3-scFv-Fc-CD28deltalck-zeta thereby replacing the HRS3-scFv by the combined HRS3scFv-BW431/26scFv. The resulting vector coding for the bispecific anti-CD30/CEA-CAR was termed #1457. The modular composed #1457 CAR and its mono-specific anti-CEA (#946) and anti-CD30 (#1138) counterparts are schematically shown in figure 1.

The retroviral vector coding for the #1457 CAR was produced according to SOP-GL-VectProd using a Galv pseudotyped envelope. In summary vector particle production was done transiently on the human embryonic kidney cell line 293T after Polyfect® mediated DNA transfection. Vector particles were pseudotyped with Galv. No vector titer was determined.

Transduction of human blood lymphocytes was done according to SOP standard techniques. In summary human lymphocytes were transduced with a 2-

day supernatant from transfected 293T-cells for 2 days. The CAR #1457 was expressed by 20-35 % of human T-cells as measured at day 2 by flow cytometry using an antibody directed to the extracellular constant IgG1 CH2 CH3 domain of the CAR.

$CD4^+$ and $CD8^+$ T-cells which express the CAR #1457 on the cell surface can be recorded by use of the 9G10 antibody which binds specifically the CAR HRS3 scFv domain. T-cells engineered with the #1457 CAR bind specifically to CD30 expressing cells and become activated indicated by increased secretion of cytokines including IFN-γ, by increase in proliferation and in cytolysis of $CD30^+$ target cells. Noteworthy, only background levels of IL-2 are secreted when T-cells are stimulated by the CAR. IL-2, however, is secreted in physiological amounts when T-cells are stimulated by their physiological TCR and CD28. Activation of the T-cells #1457 is antigen-specific as defined by the specificity of the CAR since $CD30^-$ cells do not trigger T cell activation. Soluble CD30, which accumulates in the serum of $CD30^+$ lymphoma patients, does not block CAR mediated T-cell activation in concentrations up to 10 µg/ml [Hombach A, et al., Cancer Res. 1998 Mar 15;58(6):1116-9]. This is due to the fact that the CAR must be cross-linked by binding the multiple copies of the targeted antigen in order to trigger T-cell activation which can only occur when CD30 is immobilized or expressed on the surface of target cells but does not occur when CD30 protein is present in solution.

Example 1: Activity of dual specific CAR modified T-cells toward CEA^+ and/or $CD30^+$ cell lines

Engineering of T-cells with CAR #1457 was performed as described above. The CAR was detected on the T-cell surface by flow cytometry utilizing an antibody against the Fc domain in the extracellular moiety of the CAR (Fig. 1, 2). The anti-CEA/CD30 CAR was expressed with similar efficiency as the mono-specific CARs with either the CD30 or the CEA binding domain. To test for dual specificity of the #1457 CAR, grafted T-cells were co-incubated either with $CD30^+$ or CEA^+ target cells and redirected cytotoxicity and IFN-gamma secretion were monitored. Whereas T-cells with anti-CEA and anti-CD30 CAR were only activated by CEA^+ or $CD30^+$ target cells, respectively, the bispecific CAR activates T-cells after coculture with both CEA^+ $CD30^-$ and CEA^- $CD30^+$ tumor cells. T-cell activation results in specific target cell lysis and IFN-gamma secretion (cf. Fig. 4, 5 and Example 3).

Conclusion:

T-cells with the #1457 CAR harbour dual specificity for binding, i.e., for CD30 and CEA. T-cells expressing the bispecific CAR were activated by engaging target cells with one cognate CAR antigen only as well as by target cells with both antigens.

Example 2: Specific expansion of #1457 CAR modified T-cells

T-cells were engineered with the #1457 CAR or for comparison with the corresponding single CD30 or CEA specific CAR, respectively, and cultivated in presence of IL-2 (400 U/ml). The initial number of CAR+ T-cells was 20-30%. Cells were tested at different time points for CAR expression by flow cytometry. As summarized in Figure 3, the number of T-cells with the anti-CD30-CAR and the anti-CEA/CD30 CAR #1457 increased up to 90% of T-cells during cultivation whereas the number of anti-CEA CAR T-cells did not exceed 50%.

Conclusion

Data demonstrate that CAR T-cells with the CD30-binding domain expand superior compared to CAR T-cells with a CEA binding domain. The superior effect is also present when the anti-CD30 binding domain is linked with the anti-CEA binding domain.

Example 3: Increased specific lysis by CAR #1457 engineered T-cells and CAR #1457 engineered CIK T-cells toward CD30-negative target cells

(a) T-cells

Human T-cells were engineered to express the CAR #1457, the CEA-specific CAR and the CD30-specific CAR, respectively. The same number of CAR T-cells was co-incubated with tumor cells which express either CEA or CD30 or both. Data presented in Figure 4 show that the cell line being negative for both CEA and CD30 (Colo320) was not lysed while specific lysis of LS174T cells was observed which is a $CEA^+/CD30^-$ cell line. Therein, the anti-CEA/CD30

CAR T-cells demonstrate a higher specific lysis compared to the anti-CEA CAR T-cells.

The cytolytic activity is accompanied by release of IFN-g, a marker for T-cell activation, as shown in Figure 5. Anti-CEA/CD30 CAR #1457 T-cells produced IFN-gamma when co-incubated with the $CEA^{+}/CD30^{-}$ LS174T-cells as well as with the CEA^{-} $CD30^{+}$ cells. No IFN-gamma was produced upon coincubation with CEA^{-} $CD30^{-}$ Colo320 cells. T-cells with the anti-CEA CAR or anti-CD30 CAR released IFN-gamma only upon co-incubation with CEA^{+} or $CD30^{+}$ cells, respectively.

(b) CIK cells

CIK cells were generated according to standard procedures and were engineered with the anti-CD30/CEA CAR #1457. After 8 days post transduction #1457 CAR CIK cells were co-cultivated with $CD30^{-}CEA^{+}$, $CD30^{-}CEA^{-}$ or $CD30^{-}CEA^{-}$ target cells and target cell lysis was recorded. CIK cells expressing the corresponding monospecific anti-CD30 and anti-CEA CAR, respectively, served as control. Bispecific anti-CD30/CEA and mono-specific anti-CD30 CAR T-cells lysed $CD30^{+}$ target cells with similar efficiency. In contrast, CAR #1457 CIK cells lysed CEA^{+} $CD30^{-}$ target cells more efficiently than CIK cells with the anti-CEA CAR (Fig. 6). Data indicate that CAR #1457 CIK cells surprisingly show a higher cytolytic activity against tumor cells that do not express CD30 than CIK cells with a CEA-specific CAR. <u>Conclusion:</u>

T-cells and CIK cells engineered with the CAR #1457 show enhanced anti-tumor reactivity against target cells, both which express and which lack CD30, compared to T-cells with the monospecific CAR. This is unexpected in particular for those target cells which lack CD30.

Example 4: Improved anti-tumor activity of # 1457 CAR T-cells in a xenograft mouse model.

The in vivo activity of anti-CD30/CEA CAR #1457 T-cells was monitored in the immune deficient Rag $^{-/-}$ common gamma chain $^{-/-}$ mouse. Anti-CEA-CAR and anti-CD30/CEA CAR #1457 T-cells were engineered as described above and subcutaneously co-injected in a sub-therapeutic dose ($2.5x10^{5}$ CAR T-cells/animal) with CEA^+ $CD30^-$ C15A3 tumor cells ($1x10^6$ cells/animal), that were transfected to express human CEA, into mice. Mice without T-cells and non-modified T-cells were used for control and tumor growth was monitored every 2nd-3rd day. Growth curves (Fig. 7A) and area under curve (Fig. 7B) were determined. Whereas anti-CEA-CAR T-cells produced slightly delay of tumor growth, anti-CD30/CEA CAR T-cells significantly delayed tumor growth indicating higher anti-CEA activity of the bispecific CAR T-cells #1457 in vivo than T cells with the anti-CEA CAR.

Conclusion:

Bispecific anti-CD30/CEA CAR T-cells are more effective in an prolonged anti-tumor response against CEA^+ $CD30^-$ tumor cells in vivo compared to T cells with the mono-specific anti-CEA CAR. The improved anti-tumor reactivity is due to the particular CAR design because human CD30 is neither expressed by the murine host nor by the grafted mouse tumor cells and not recognized on tumor cells or on cells of the host.

Example 5: Improved anti-tumor activity of anti-CD30/CEA #1457 CAR but not anti-CD25CEA #1576 CAR.

The bispecific #1576 CAR of Seq. ID. No. 18 encoded by the sequence of Seq. ID. No. 17 with specificity for both the lymphocyte activation antigen CD25 and CEA was generated by substituting the HRS3 scFv with the anti-CD25 scFv RFT5 (Fig. 8A; cf Fig. 1). Engineering of T-cells with CAR #1576 was performed as described above by flanking the cDNA for the anti-CD25 scFv with overlapping sequences and sequences for appropriate restriction enzymes by PCR. The sequence of the HRS3 scFv was substituted and the resulting #1576 CAR expressed by transduction with retroviral supernantant according to transductionSOP. The CAR was detected on the T-cell surface by flow cytometry utilizing an antibody against the Fc domain in the extracellular moiety of the CAR. The anti-CD25/CEA CAR was expressed with similar efficiency than monospecific and bispecific anti-CD30 CARs, respectively. Specific CAR T cell activation and lysis of CEA+ target cells was tested by co-cultivation with CEA+ and CEA- tumor cells respectively. All CARs lysed specifically CEA+ target cells but the anti-CD30/CEA CAR lysed tumor cells with higher efficiency than monospecific and bispecific anti-CEA and anti-CD25/CEA CARs, respectively (Fig. 8B).

Conclusion:
T-cells with the #1457 anti-CD30/CEA CAR but not with the #1576 anti-CD25/CEA CAR were activated against CEA+ target cells with higher efficiency than anti-CEA-monospecific CAR T cells.

Example 6: Bispecific anti-CD30 antibodies with different second specificity

Bispecific anti-CD30 CAR molecules were generated as described in Example 1 except for substituting the anti-CEA scFv moiety with scFv moieties with specificity against MUC1, C595-scFv, resulting in anti-CD30/MUC1-CAR #1587, and with specificity against TAG-72, B72.3-scFv, resulting in anti-CD30/TAG-72 CAR #1650. The sequences thereof are Seq. Id. Nos. 20 and 19 for #1587 and Seq. ID. Nos. 22 and 21 for #1650.

Figure 9A is a schematic representation of these bispecific CARs. Figure 9B demonstrates expression of these CAR molecules on transfected 293 T-cells.

Claims

1. A recombinant polypeptide containing at least the following domains starting from the N-terminus to the C-terminus: a first domain containing an anti-CD30 single chain antibody unit, in particular, HRS3 scFv of SEQ ID No. 2 or homologs thereof having at least 70% identity with SEQ ID No. 2 binding specifically to CD30, and an antibody unit said antibody unit being specific for an antigen present on the surface of a predetermined target cell, in particular, being specific for a tumor-associated antigen; optionally a spacer domain; a trans-membrane domain; and a cytoplasmatic signalling domain.

2. The recombinant polypeptide according to claim 1 wherein the order in the first domain is from the N-terminus to the C-terminus: i) an anti-CD30 single chain antibody unit and an antibody unit, said antibody unit being specific for an antigen present on the surface of a predetermined target cell, or ii) an antibody unit whereby said antibody unit being specific for an antigen present on the surface of a predetermined target cell, and an anti-CD30 single chain antibody unit.

3. The recombinant polypeptide according to claim 1 or 2 further comprising a leader sequence being located N-terminally to the first domain containing the anti-CD30 single chain antibody unit and the antibody unit whereby said antibody unit being specific for an antigen present on the surface of a predetermined target cell.

4. The recombinant polypeptide according to any one of the preceding claims wherein the anti-CD30 single chain antibody unit is the HRS3 scFv peptide of the SEQ ID No. 2.

5. The recombinant polypeptide according to any one of the preceding claims wherein the spacer domain is an IgG1 CH2 CH3 domain of SEQ ID No. 6

or homologs thereof having at least 70% identity therewith, preferably, the spacer domain is a mutated IgG1 CH2 CH3 domain according to SEQ ID No. 6.

6. The recombinant polypeptide according to any one of the preceding claims wherein the trans-membrane domain is derived from CD28.

7. The recombinant polypeptide according to any one of the preceding claims wherein the intracellular domain contains a CD3zeta or a Fc-epsilon receptor I-gamma signalling chain or a costimulatory unit, like wherein the intracellular domain is the CD3zeta signalling unit of SEQ ID No. 10 or a homolog thereof having at least 70% homology, or like wherein the intracellular domain is the IgE Fc epsilon receptor-I gamma-signalling unit of SEQ ID No. 12 or a homolog thereof having at least 70% homology.

8. The recombinant polypeptide according to any one of the preceding claims wherein the intracellular domain is a CD28 signalling domain, in particular, a CD28 domain lacking the LCK binding motive, like the domain of SEQ ID No. 8.

9. The recombinant polypeptide according to any one of the preceding claims wherein the antibody unit binds to a tumor-associated antigen including any one of carcinoembryonic antigen (CEA), CA19-9, CA72-4 (TAG-72), PSCA, Muc-1, HMW-MAA, p97 melanotransferrin, fetal actelycholin receptor, ErbB2 (Her2/neu), multi-drug-resistance protein (MDR), CD19, CD20, TOSO.

10. The recombinant polypeptide according to any one of the preceding claims wherein the antibody unit binds to viral antigens, in particular, heptatis virus B-associated antigen S or L, cytomegalovirus-associated antigen.

11. A nucleic acid molecule comprising a nucleic acid sequence encoding the polypeptide according to any one of claims 1 to 10, like the nucleic acid sequence of SEQ ID No. 13.

12. A vector comprising the nucleic acid sequence according to claim 11, in particular, a viral vector.

13. A cell, cell line or host cell containing a vector according to claim 12 or a nucleic acid molecule according to claim 11.

14. The cell, cell line or host cell according to claim 13 being modified peripheral blood cells, in particular, being lymphocytes including T-cells, like $CD8^+$ or $CD4^+$ T-cells.

15. Lymphocyte, in particular, a T-cell, like a $CD8^+$ $CD4^+$ T-cell, expressing on its surface chimeric antigen receptors having in their extracellular domain an antibody unit, said antibody unit being specific for an antigen present on the surface of a predetermined target cell, and an anti-CD30 single chain antibody unit expressed by said cells whereby said units are either present in a single polypeptide as defined in any one of claims 1 to 10 or present in different functional chimeric antigen receptors.

16. A cell according to any one of claims 13 to 15 for use in treating cancer, in particular, $CD30^-$ cancer, whereby said cancer is a cancer expressing the antigen to which the anti-tumor antigen antibody is directed to.

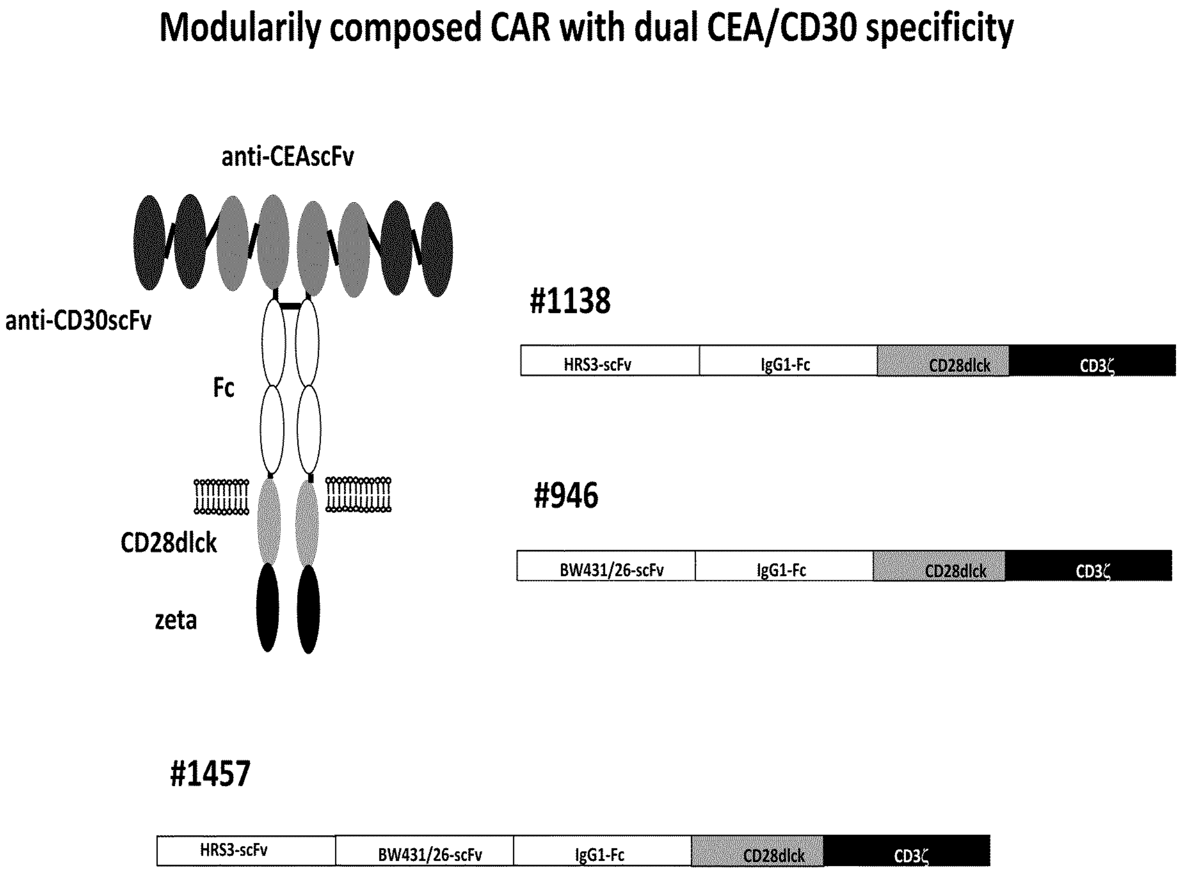


Figure 1

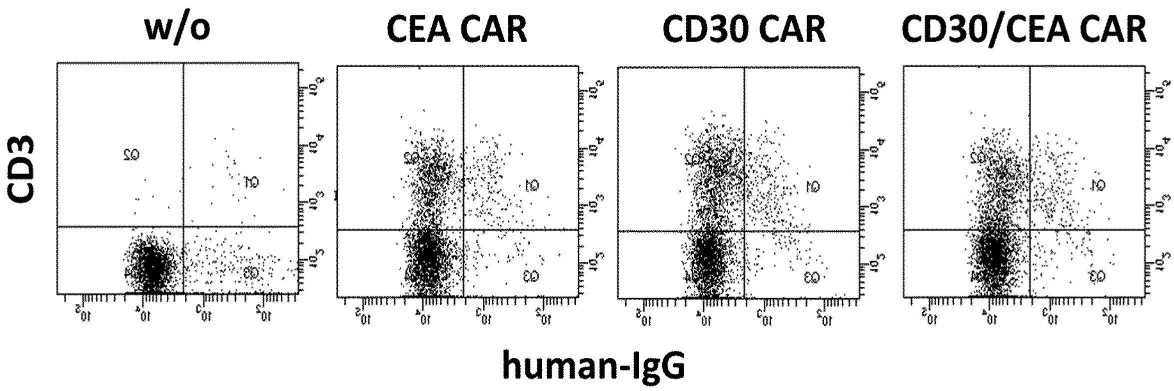


Figure 2

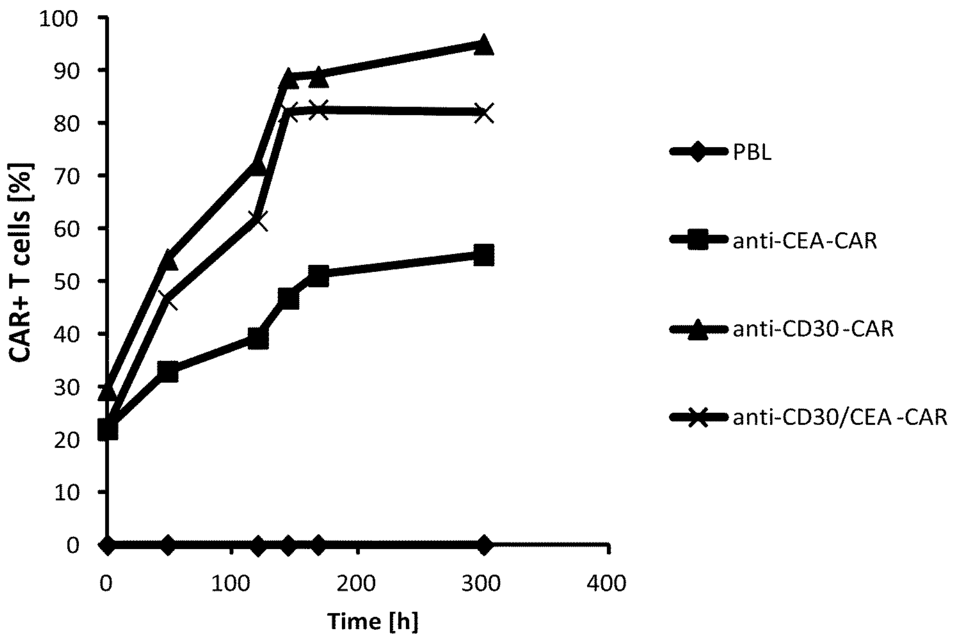


Figure 3

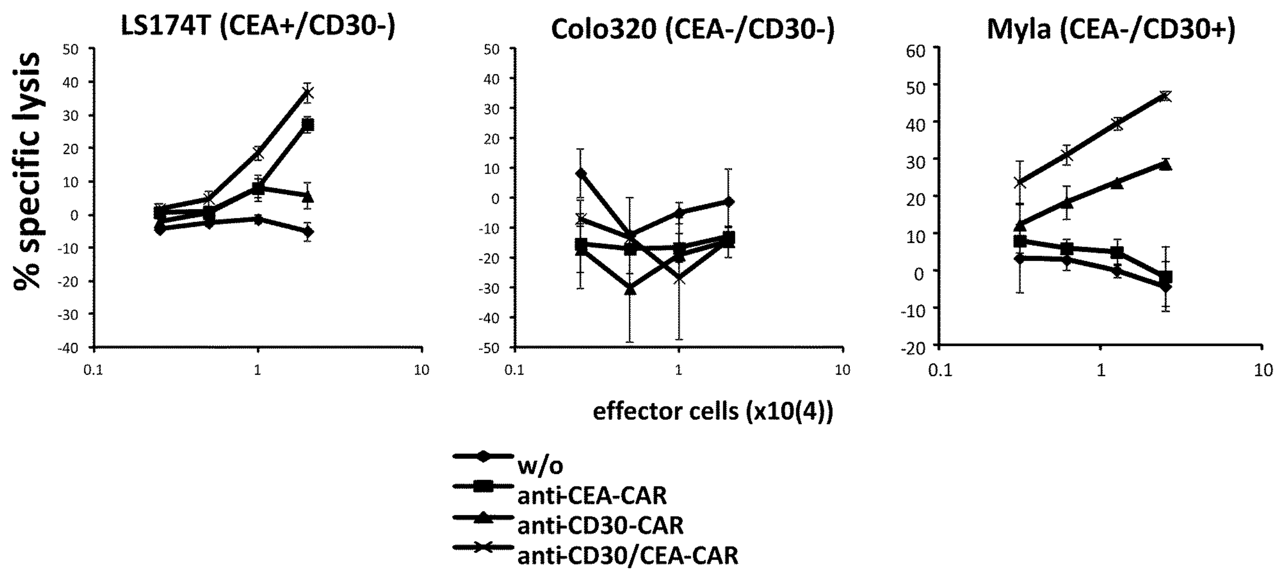


Figure 4

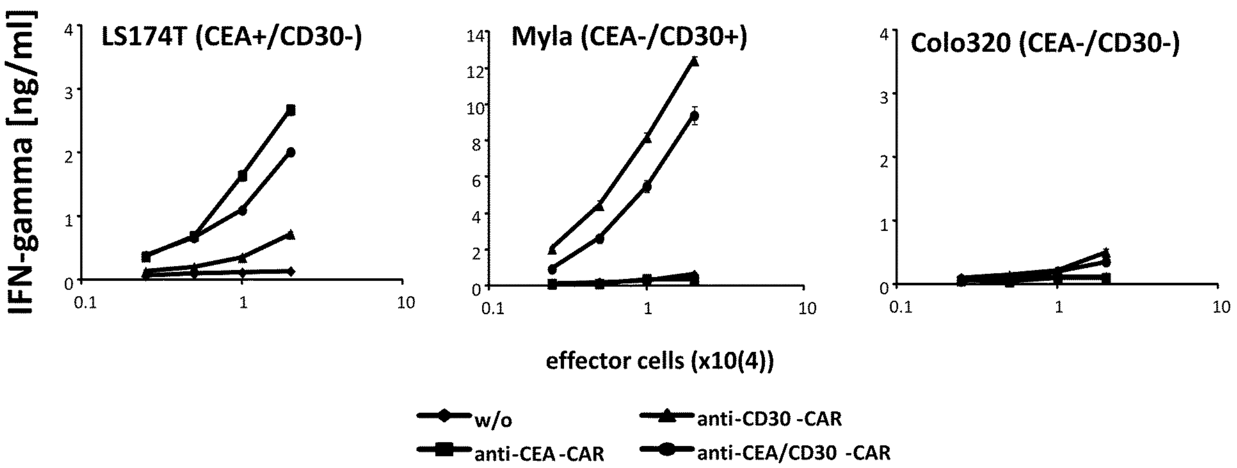


Figure 5

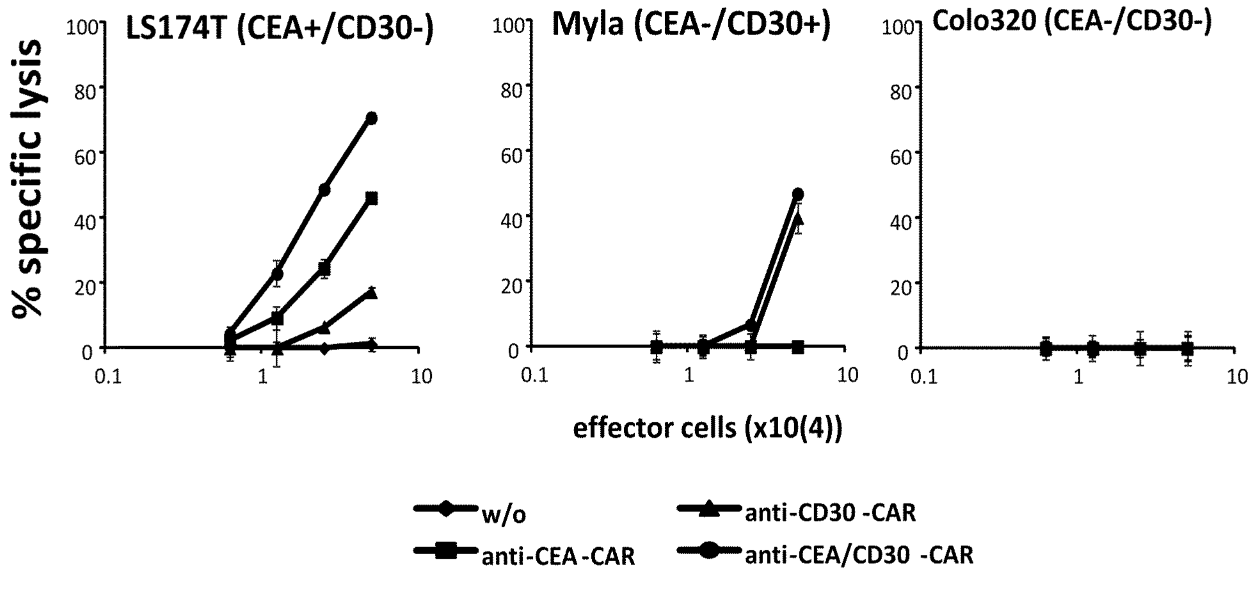


Figure 6

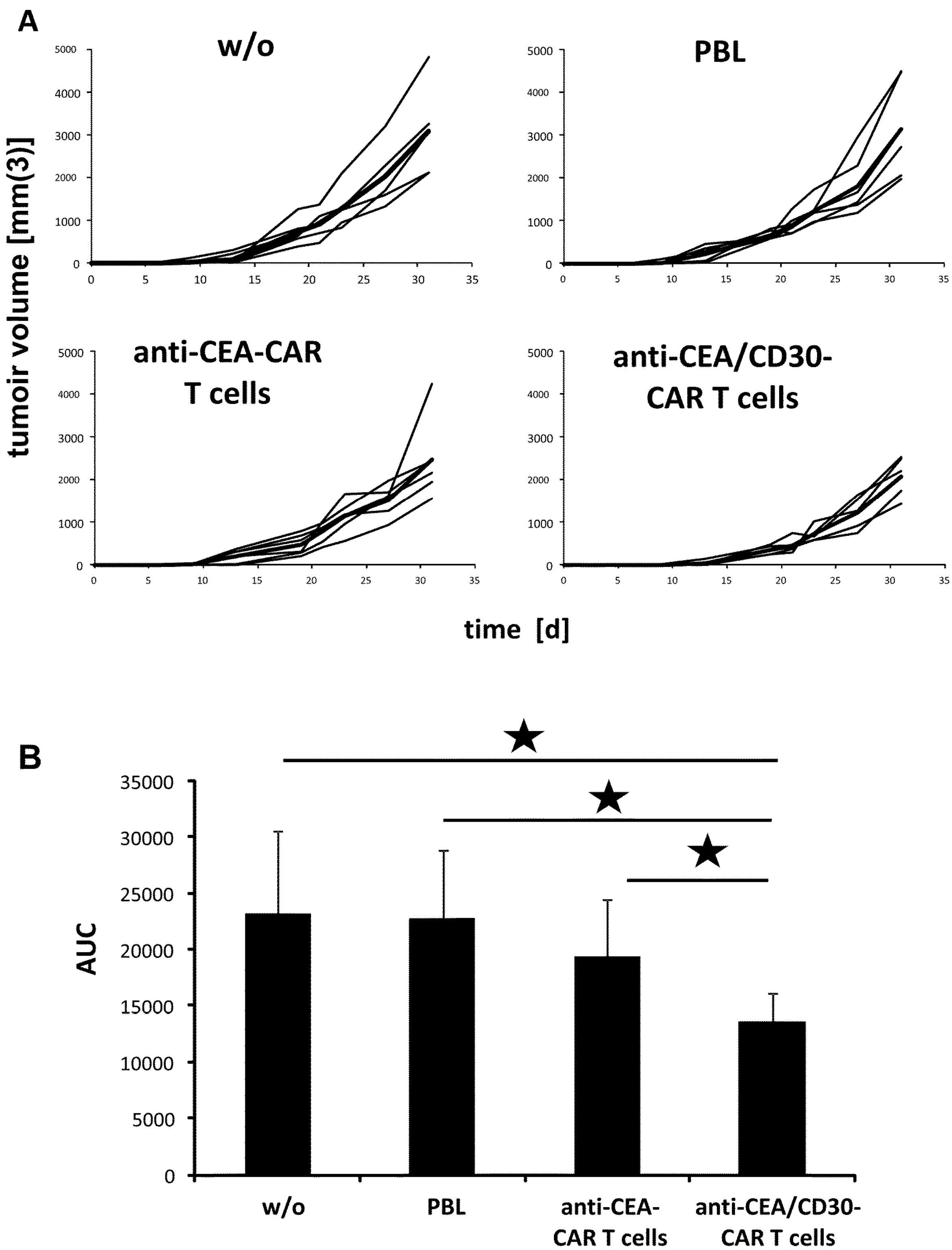
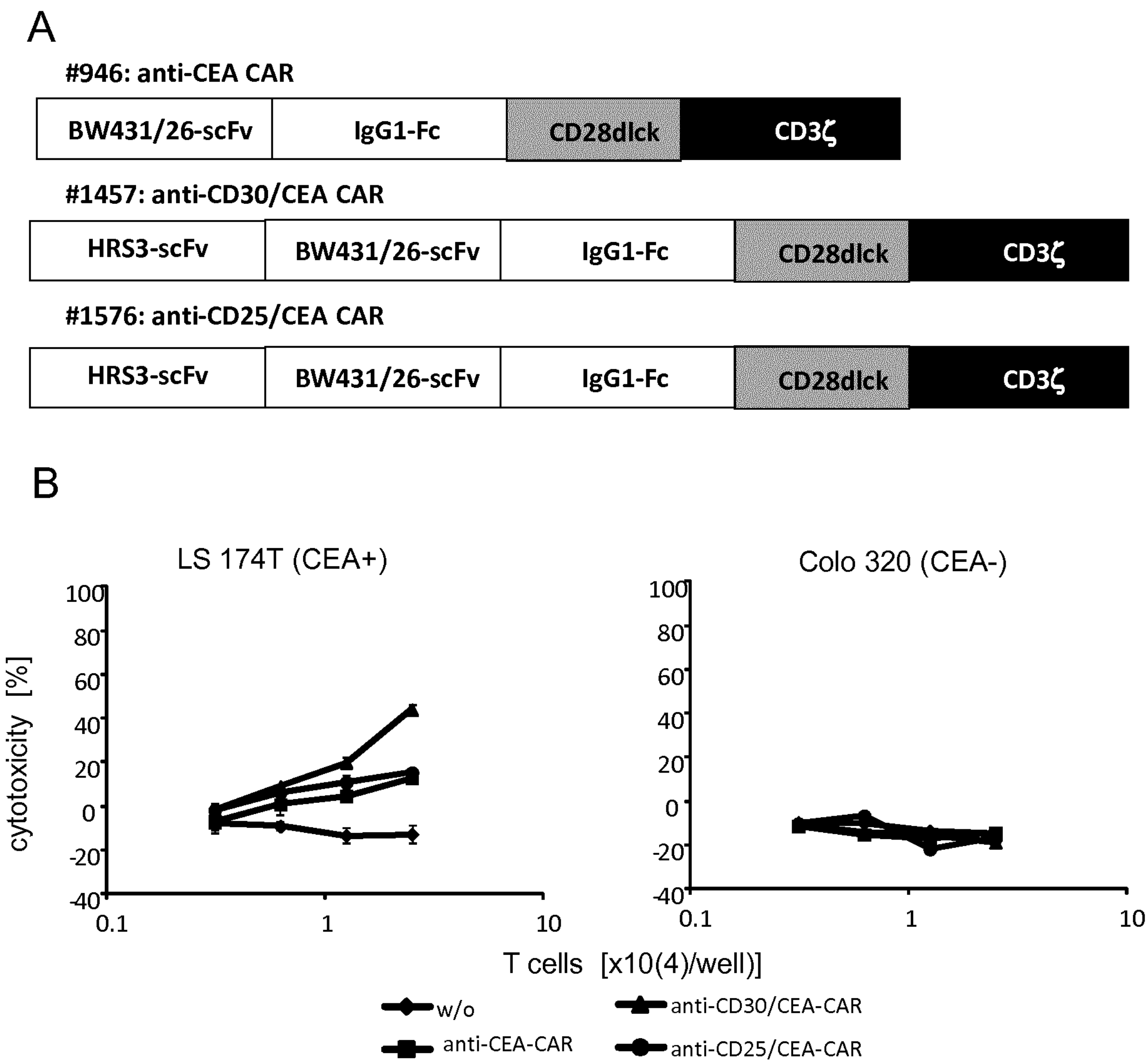

A
w/o
PBL
anti-CEA-CAR
T cells
anti-CEA/CD30-
CAR T cells
tumoir volume [mm(3)]
time [d]
B
AUC
w/o
PBL
anti-CEA-
CAR T cells
anti-CEA/CD30-
CAR T cells

Figure 7


A
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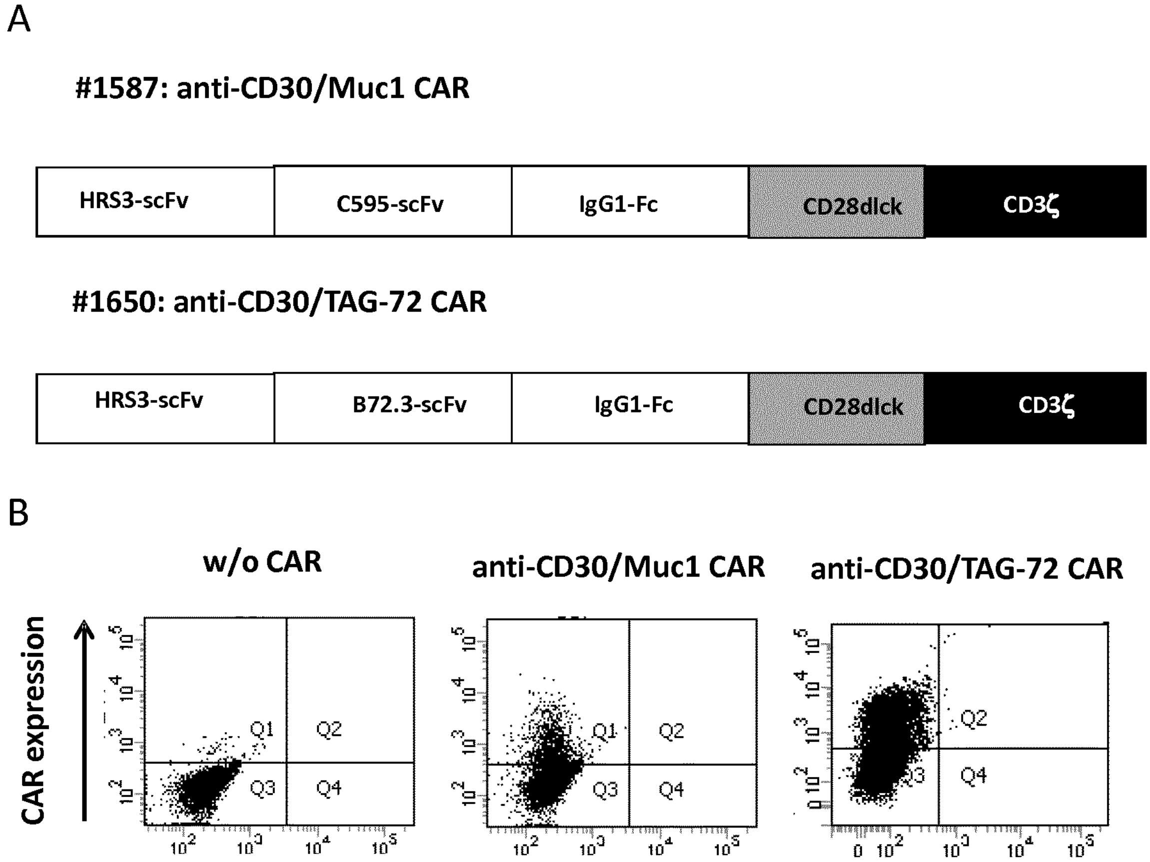
BW431/26-scFv
IgG1-Fc
CD28dlck
CD3ζ
#1457: anti-CD30/CEA CAR
HRS3-scFv
BW431/26-scFv
IgG1-Fc
CD28dlck
CD3ζ
#1576: anti-CD25/CEA CAR
HRS3-scFv
BW431/26-scFv
IgG1-Fc
CD28dlck
CD3ζ
B
LS 174T (CEA+)
Colo 320 (CEA-)
cytotoxicity [%]
100
80
60
40
20
0
-20
-40
0.1
1
10
T cells [x10(4)/well)]
w/o
anti-CD30/CEA-CAR
anti-CEA-CAR
anti-CD25/CEA-CAR

Figure 8


A
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HRS3-scFv
C595-scFv
IgG1-Fc
CD28dlck
CD3ζ
#1650: anti-CD30/TAG-72 CAR
HRS3-scFv
B72.3-scFv
IgG1-Fc
CD28dlck
CD3ζ
B
w/o CAR
anti-CD30/Muc1 CAR
anti-CD30/TAG-72 CAR
CAR expression
Q1
Q2
Q3
Q4

Figure 9

INTERNATIONAL SEARCH REPORT

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:

   a. [X] forming part of the international application as filed:

      [X] in the form of an Annex C/ST.25 text file.

      [ ] on paper or in the form of an image file.

   b. [ ] furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. [ ] furnished subsequent to the international filing date for the purposes of international search only:

      [ ] in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      [ ] on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2015/066252

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K16/28 C07K14/725 C07K16/30
ADD. A61K39/00

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)
C07K A61K

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

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

EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	SCOTT WILKIE ET AL: "Dual Targeting of ErbB2 and MUC1 in Breast Cancer Using Chimeric Antigen Receptors Engineered to Provide Complementary Signaling", JOURNAL OF CLINICAL IMMUNOLOGY, KLUWER ACADEMIC PUBLISHERS-PLENUM PUBLISHERS, NE, vol. 32, no. 5, 17 April 2012 (2012-04-17), pages 1059-1070, XP035113362, ISSN: 1573-2592, DOI: 10.1007/S10875-012-9689-9 RH paragraph, p. 1068 -----	1-16

-/--

[X] 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
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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ZAKARIA GRADA ET AL: "TanCAR: A Novel Bispecific Chimeric Antigen Receptor for Cancer Immunotherapy", MOLECULAR THERAPY-NUCLEIC ACIDS, vol. 2, no. 7, 2 July 2013 (2013-07-02), page e105, XP055152438, DOI: 10.1038/mtna.2013.32 see p. 4 , last sentence of the second par., in par. of the RH column -----	1-16

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