



(51) International Patent Classification:

C07K 16/30 (2006.01) A61K 31/00 (2006.01)
A61K 39/00 (2006.01) C07K 14/725 (2006.01)
A61K 39/395 (2006.01) A61P 35/00 (2006.01)

TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

(21) International Application Number:

PCT/EP2019/065822

Published:

- with international search report (Art. 21(3))
- with sequence listing part of description (Rule 5.2(a))

(22) International Filing Date:

17 June 2019 (17.06.2019)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

18180026.9 27 June 2018 (27.06.2018) EP

(71) Applicant: ALBERT-LUDWIGS-UNIVERSITÄT FREIBURG [DE/DE]; 79098 Freiburg (DE).

(72) Inventors: CATHOMEN, Toni; Komturstr. 39a, 79106 Freiburg (DE). ALZUBI, Jamal; Komturstr. 32, 79106 Freiburg (DE). DETTMER, Viviane; Habichtweg 1, 79110 Freiburg (DE). WOLF, Philipp; Eberbächle 14, 79350 Sexau (DE). SCHULTZE-SEEMANN, Susanne; Alte Str. 2, 79249 Merzhausen (DE). KUCKUCK, Irina; Willy-Brandt-Allee 8, 79111 Freiburg (DE). ABKEN, Hinrich; Gotthard-Bauer-Str. 1, 94333 Geiselhöring (DE). KÜHLE, Johannes; Platenstr. 45, 50825 Köln (DE).

(74) Agent: KELLER, Günter et al.; Unsöldstr. 2, 80538 München (DE).

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

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM,

(54) Title: CHIMERIC ANTIGEN RECEPTORS THAT BIND TO PROSTATE SPECIFIC MEMBRANE ANTIGEN

(57) Abstract: The present invention relates to a novel chimeric antigen receptor (CAR) comprising an antigen-binding fragment which binds specifically to PSMA antigen, and a method of manufacturing high-quality CAR T cell products by transfection and/or transduction of T cells therewith, which allows to eradicate tumors in vivo alone or in combination with pharmaceutical drugs, such as chemotherapies, biopharmaceutical drugs, such as antibodies, or small-molecule drugs, such as protein kinase inhibitors.

Chimeric antigen receptors that bind to prostate specific membrane antigen

The present invention relates to chimeric antigen receptors that bind to tumor antigens whereby the antigen is the prostate specific membrane antigen (PSMA). The chimeric antigen receptors (in the following CAR) are brought into immune cells, in particular T cells, NK cells, iNKT cells and CIK cells, which then specifically react with tumor cells expressing PSMA which leads to the elimination of the tumor cells. The constructs of the present invention contain two major parts. On the one hand the antigen-binding region which specifically binds to the prostate specific membrane antigen (PSMA) and on the other hand co-stimulatory domains derived from a receptor of an immune cell responsible for signal transduction and activation of the immune cell.

Prostate cancer remains the second-most frequently diagnosed cancer among men worldwide with estimated 1.1 million new cases per year. Moreover, with expected 307,000 deaths, it represents the fifth leading cause of cancer deaths. Whereas primary tumors can successfully be treated, there is no curative treatment for advanced stages. Therefore, new therapeutic options are urgently needed.

The prostate specific membrane antigen (PSMA) is the best characterized antigen in prostate cancer for antibody-based diagnostic and therapeutic intervention. This protein is also known as glutamate carboxypeptidase II (EC 3.4.17.21), N-acetyl-linked acidic dipeptidase I (NAALADase), or folate hydrolase. PSMA is a type II membrane glycoprotein consisting of 750 amino acids (aa) with a small intracellular domain of 19 aa, a transmembrane domain of 24 aa, and a large extracellular domain of 707 aa. The extracellular domain folds into three distinct domains: the protease domain (aa 57–116 and 352–590), the apical domain (aa 117–351), and the C-terminal domain (aa 591–750). It shows a high structural similarity and identity to the human transferrin receptor 1. PSMA is highly restricted to the surface of prostate cancer cells, is present on cancer cells during all tumor stages, and shows an enhanced expression in androgen-independent and metastatic disease. PSMA is not secreted into the extracellular space and undergoes constitutive internalization, which is enhanced by binding of PSMA-specific antibodies.

These characteristics make it an ideal candidate for the targeted treatment of advanced prostate cancer. Moreover, PSMA was also found to be expressed in the neovascular endothelium of virtually all solid tumor types without expression in normal vascular endothelium. It is therefore considered to be a unique antiangiogenic target.

Monoclonal antibodies (mAbs) are highly specific and versatile tools for cell targeting. In the last decades, they have attracted high interest in medical research and have become the most rapidly expanding class of pharmaceuticals for treating a variety of human diseases including cancer. Antibody 7E11 was the first published PSMA-specific mAb and was found to bind to the N-terminus (MWNLLH) of the intracellular domain of PSMA. The In-labeled form of 7E11 (ProstaScint, Cytogen, Philadelphia, PA) has received approval from the U.S. Food and Drug Administration (FDA) for the detection and imaging of metastatic prostate cancer in soft tissues. However, because the antibody binds an intracellular epitope, 7E11 is not capable to bind to viable cells. Positive signals in the *in vivo* imaging with ProstaScint had to be traced back to the detection of dead or dying cells within the tumor masses. Therefore, a new class of anti-PSMA mAbs was generated, which specifically bind to extracellular epitopes of PSMA expressed by living cells.

EP 1 883 698 discloses three different mAbs, 3/A12, 3/E7, 3/F11, which show a strong and specific binding to the extracellular moiety of PSMA on the surface of prostate cancer cells and prostate tissue specimens. In direct comparison with mAb J591, a clinically validated antibody for radioimmunotherapy (PMID:18552139; PMID:24135437; PMID:25771365; PMID: 26175541), the mAb 3/F11 showed higher binding to PSMA expressing C4-2 prostate cancer target cells (K_d [defined as mean half-maximal saturation concentration] of 3/F11 = 9 nM; K_d of J591 = 16 nM). Moreover, competitive binding studies demonstrated that mAb 3/F11 binds to a different extracellular PSMA epitope than J591 (PMID:19938014). In an immunohistological study on a panel of human normal tissues, no binding of the 3/F11 mAb to PSMA-negative tissues (adrenal, bone marrow, cerebellum, cerebrum, pituitary, colon, esophagus, heart, kidney, liver, lung, mesothelial cells of the pericardium, nerve, ovary, pancreas, skeletal muscle, skin, spleen, stomach, testis, thymus, thyroid, tonsil, and uterus) was detected. Only binding to secretory cells of the salivary glands and to duodenal brush border cells was observed, which are known to express PSMA (PMID:19938014). The mAb 3/F11 showed a moderate immunoreactivity on acinar secretory epithelial cells of all tested normal prostate tissues. A more intense and extensive staining was noticed in nearly all epithelial cells of adenocarcinomas as well

as in lymph node metastases. No immunohistological staining on frozen sections of breast specimen was detected. In contrast and in accordance with other published data, staining of the mammary ductal epithelium was detected with mAb J591. Since PSMA expression in the breast tissue was neither detected by PCR nor by Western blotting, it is likely that mAb J591 cross-reacts with another antigen.

The single-chain variable fragment (scFv) D7 (as disclosed in EP 1 883 698 B1) was generated by phage display technique from the mAb 3/F11. The most important fragments for the specificity of the anti-PSMA scFv are the V_L and the V_H parts, which are preferably linked with a polyglycin linker. D7 bound to PSMA-expressing C4-2 cells with a K_d of about 18 nM, and preincubation with the parental mAb 3/F11 fully inhibited the binding activity. This proved that the scFv D7 binds to the same PSMA epitope as 3/F11. The scFv D7 and a humanized version thereof were successfully used for the construction of *Pseudomonas* Exotoxin A (PE) based immunotoxins, which showed high and specific cytotoxicity against PSMA-expressing prostate cancer cells and *in vivo* antitumor activity in mice bearing prostate tumors.

According to the present invention the D7 scFv was used for the construction of chimeric antigen receptors (CAR's) to provide constructs for use in immune cells for targeting cancer cells expressing PSMA.

Ma et al. [The Prostate (2014) **74**, pp 286-296] disclose a second generation CAR against PSMA comprising a CD28 costimulatory domain and CD3 ζ signaling domains and an antigen binding part from a mouse anti-human PSMA monoclonal antibody 3D8 which is commercially available from Northwest Biopharmaceutics, Inc.

Santoro et al. [Cancer Immunol. Res. (2015), pp 68-84] describe T-cells bearing a chimeric antigen receptor against prostate-specific membrane antigen, whereby the PSMA binding portion is derived from the antibody J591.

Zhong et al. [Molecular Therapy (2010), pp. 413-420] disclose also chimeric antigen receptors whereby the PSMA binding fragment is also derived from the antibody J591. The sequence of J591 is well-known in the art. WO 2009/017823 discloses the V_H and V_L domains thereof.

The amino acid sequence of the antigen-binding fragment D7 (Figure 10; SEQ ID NO:1) and the nucleic acid sequence (SEQ ID NO:8), including complementary strand coding for said construct (SEQ ID NO:9), is provided. Moreover, the highly important parts of the antigen-binding fragment, namely the CDRs: (CDR-H1 (SEQ ID NO:2), CDR-H2 (SEQ ID NO:3), CDR-H3 (SEQ ID NO:4), CDR-L1 (SEQ ID NO:5), CDR-L2 (SEQ ID NO:6) and CDR-L3 (SEQ ID NO:7)) are shown and highlighted by grey arrows.

Humanization of murine antibodies involves the transfer of beneficial properties (e.g. antigen-specific binding, avoidance of off-target effects by non-crossreactivity with other antigens) from one antibody to another to reduce immunogenicity. Humanization is usually necessary for human use as patients typically respond with an immune reaction against non-human antibodies that can lead to ineffectiveness of treatment and, in the worst-case scenario, to a life-threatening situation. A humanized construct can be derived from the sequence of the antigen-binding fragment shown in Figure 10. The CDR regions structurally define the paratope, that is, the contact site of the antigen-binding fragment with the antigen. The remainder of the sequence codes for the framework regions, which form the scaffold of the paratope. For the humanization process (e.g. by *in silico* modeling), the framework sequence is first compared with other antigen-binding sequences derived from humans. Usually a human sequence (acceptor framework) is selected which has the highest similarity with the framework sequence shown in Figure 10. The CDR regions are grafted into the human acceptor framework to eliminate amino acid sequences which may cause undesired human anti-mouse-antibody (HAMA) immune reactions. Substitutions at potentially critical positions (e.g. amino acids responsible for folding the paratope or the VH-VL interface) are analyzed for prospective back mutations. Even in the CDR sequences exceptional modifications of amino acids may be made to avoid immunogenicity, to ensure the right folding of the paratope, and to maintain the antigen-specific binding.

In the course of the humanization of antibodies preferably a sequence is selected among human immune sequences which have the highest homology with the corresponding murine sequence. Then the location of the CDRs is determined. The determination of the CDRs is well-known in the art and it should be noted that different methods for the determination are known whereby it is possible that the locations of the CDRs differ somewhat. In the course of the present invention the determination of the CDRs according

to Kabat was used and also the determination according to the IMGT (International Immunogene Ticks).

In a preferred embodiment of the present invention the humanization was performed according to the so-called "CDR-grafting". The functional CDRs are determined preferably according the IMGT method and those CDRs are transferred in a human framework region which has the highest sequence homology to the starting murine antibody. Then the differences regarding the single amino acids in the framework region of the humanized and murine antibodies were determined with regard to the biochemical properties like size, polarity or charge. In the first step similar amino acids were adapted and successively the different amino acids were changed in order to end up with a complete human framework.

Since the humanized versions disclosed herein have maintained the CDRs either completely or to a very large extent the humanized variants have the same function of the murine antibody whereby, however, the affinity may differ somewhat from each other.

The results of the humanization experiments are shown in Figure 11. It turned out that the CDR-H1, CDR-H3 and CDR-L3 should be maintained without any amendment. In the CDR-L2 one amino acid can be replaced which is indicated as X9. X9 may have the meaning of an aliphatic, uncharged amino acid which can be glycine, alanine, valine, lysine, isoleucine and/or proline.

In CDR-L1 two amino acids can be replaced which are designated as X7 and X8. Those amino acids can be hydrophilic, uncharged amino acids such as serine, threonine, asparagine and/or glutamine.

The highest flexibility seems to have CDR-H2 wherein up to six amino acids can be replaced. Those amino acids are designated as X1, X2, X3, X4, X5 and X6. The amino acids used for replacement in the humanized antibodies or antigen-binding fragments have the following meaning:

X1, X4, X6,: hydrophilic, uncharged amino acids [serine (S), threonine (T), asparagine (N), glutamine (Q)]

X2, X3,: aliphatic, uncharged amino acids [glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), proline (P)]

X5: basic amino acids [histidine (H), lysine (K), arginine (R)].

In recent years, adoptive immune cell therapy has been introduced as a novel concept to treat different cancers by redirecting the immune system to eliminate the tumor cells. One of the most successful concepts is based on the genetic engineering of T cells to express chimeric antigen receptors (CARs) that bind tumor antigens or tumor-associated antigens in a human leukocyte antigen (HLA)-independent manner. CD19 targeting CAR T cells have been successfully used to treat B cell acute lymphoblastic leukemia (B-ALL), with >90% of patients going into complete remission in several clinical trials. Based on this success, more than 200 clinical trials have been initiated to treat mostly hematological malignancies. For solid tumors, however, the potency of CAR T cell therapy seems rather low to date. The main reason for this failure seems to be the tumor microenvironment (TME), which is the cellular environment in which the tumor exists. It includes various kinds of immune cells, fibroblasts, the extracellular matrix (ECM) as well as the surrounding blood vessels. Many mechanisms that describe restriction of cytotoxic T cell activity in the TME have been described, including the activation of PD-1 based T cell immune checkpoint inhibition. Overcoming these restrictions, combined with T cell checkpoint antagonists, will help to improve anti-tumor activity in the TME.

The tumor eradication, as used in the present invention, requires an adequate survival and intratumoral activation of tumor antigen-specific immune cells, preferably T cells. To meet these requirements T cells must be given appropriate activating signals at the time of antigen-priming and stimulation. The chimeric antigen receptors of the present invention combine therefore an antigen-binding fragment as part of the receptor on T cells. The antigen-binding fragment binds to the specific antigen (here PSMA) to which the T cells should bind. Moreover, the receptors contain sequences from CD28 and 4-1BB, respectively, as co-stimulatory signaling domains. It has been shown that the addition of CD28 sequences, or other co-stimulatory signaling domains, to CD3 ζ chain-based receptors increases antigen-induced secretion of interleukin-2 and in vitro T cell expansion. In the present case the signaling domain consists of the CD3 ζ domain and either the intracellular CD28 or the 4-1BB domain.

In general, the design of a CAR can vary and meanwhile several generations of CARs are known. The main components of a CAR system are the CD3 ζ intracellular domain of the T cell receptor (TCR) complex, the transmembrane domain, the hinge region and the

antigen-binding part. In the design of a CAR, the antigen-binding domain is linked to a hinge region, which is also called a spacer region, the transmembrane domain, and a cytoplasmic domain. Those parts are responsible for the position of the antigen-binding part, the attachment in T cell membrane, and intracellular signaling. Besides this structural rule in the CAR design the morphological characteristics of the hinge region, such as their length and sequence, are important for an efficient targeting. The intracellular domain acts as a signal transducer. The cytoplasmic segment of the CD3 ζ plays the principal rule due to different functions in activated T cells and the resting ones. However, this cytoplasmic part cannot activate the resting T cells alone. Therefore, there is a need of at least a secondary signal for the full activation of T cells. In the present invention, preferably 4-1BB or CD28 co-stimulatory domains were used. Other co-stimulatory domains, such as co-stimulatory domains derived e.g. from CD27, ICOS and OX40, can be used alternatively.

In preferred embodiments mutations are introduced into the human IgG1 Fc hinge region whereby side effects like preventing Lck activation or an unintended initiation of an innate immune response are avoided. One of those mutations avoids Lck binding and another mutation may inhibit the binding of Treg cells to the construct. Such mutations may improve the biological activity of the construct.

For the treatment of human patients, T cells have to be enriched from the individual patient's peripheral blood (autologous setting) or provided by a donor (allogeneic setting). This can be done for example by leukapheresis. The enriched T cells are then transfected or transduced ex vivo with a suitable vector comprising the genetic information for the CAR.

The genetic information coding for the CAR is inserted into a suitable vector. Such vectors are preferably lentiviral or retroviral vectors. A gold standard for transduction of primary T cells are presently considered lentiviral vectors which seem to be a valid alternative to simpler retroviral vectors. As a further alternative to lentiviral vectors the information can be introduced into the T cells with the help of transposons or plasmids. An alternative to both viral and non-viral delivery are the recently described gene editing tools, designated as CRISPR/Cas or other designer nucleases, such as transcription activator-like effector nucleases (TALENs) or zinc finger nucleases (ZFNs). This technology platforms offer the possibility to target virtually any genomic site in a targeted manner. In the case of CRISPR/Cas, the editing complex comprises a Cas nuclease and a guide RNA, usually

composed of a CRISPR RNA (crRNA) and a transacting crRNA. Upon hybridization of the guide RNA to the target sequence, Cas9 (or another Cas nuclease, such as e.g. Cpf1/Cas12a) generates a double-strand break, which can be repaired by non-homologous end joining (NHEJ), an event that can result in a loss of function of the genomic locus. In the presence of a suitable donor DNA, by a mechanism of homology-directed repair (HDR), an exogenous sequence (CAR sequence) can be introduced into the targeted locus. This can be exploited to deliver CAR expression cassette in a desired genomic locus that does not interfere with endogenous gene function and therefore minimizing the genotoxic effects experienced with integrating viral vectors. In a further preferred embodiment, genome editing is used to place the CAR coding sequence under control of an endogenous promoter. The expression from an endogenous promoter, such as the promoter of the *TRAC* locus, could ensure optimal expression levels of the CAR construct to fulfill its function. The nucleic acid sequence coding for the CARs of the present invention is preferably optimized for the human codon usage. Particularly preferred embodiments are SE ID NO:10 coding for the CAR28 construct and SEQ ID NO:11 coding for the CAR41 construct.

In another preferred embodiment of the present invention the RNA coding for the CAR construct is introduced into the target cells, such as T-cells. Nucleic acid coding for the CAR construct may be introduced into the target cells by physical procedures, such as electroporation, or by fusion of the cells' membranes with suitable vesicles. In this embodiment, the CAR construct is preferably transiently expressed in the T-cells. The advantage is that there is then a population of transduced T-cells which is present in the treated patient only for a transient time.

In other preferred embodiments the CAR construct is introduced into natural killer cells (NK), invariant natural killer T-cells (iNKT), diverse natural killer cells (dNKT), cytokine-induced killer cells (CIK) or $\gamma\delta$ T-cells. Furthermore, suitable allogenic cells may be used.

In a further preferred embodiment, in addition to the CAR construct described herein, another transgene that modulates the immune system, such as genes coding for cytokines, chemokine receptors and/or checkpoint inhibitors, may be introduced in the immune cells. In a further preferred embodiment, genome editing is used to disrupt the expression of genes that modulate the immune system, such as genes coding for cytokines, chemokine receptors and/or checkpoint inhibitors.

In another embodiment the constructs according to the present invention and immune cells containing such constructs can be used for focal therapy with a targeted tumor injection. In this embodiment, which is performed preferably with automated devices that apply the CAR T-cells to certain places in the body of the patient, where local tumor areas are located. With a biopsy needle a sample is then withdrawn whereby a small cavity is formed. In this cavity the transduced or transfected T-cells are introduced and then the needle is withdrawn. This embodiment is particularly advantageous when there are solid tumors which are extremely difficult to treat with regular methods.

The chimeric antigen receptors disclosed herein can be used for the treatment of diseases which are related to the expression of PSMA. PSMA is expressed in tumor cells derived from prostatic cancer. There are several stages of prostatic cancer known, but it seems that PSMA is one of the markers best suited for the treatment of prostate cancer. The term "prostate cancer" comprises all forms of prostate cancer cells either derived from a primary tumor or from a metastatic tumor or from circulating tumor cells. In a particularly preferred embodiment immune cells engineered with the chimeric antigen receptors of the present invention are used against the neovascularization of solid tumors expressing PSMA.

In a further embodiment of the present invention immune cells engineered with the chimeric antigen receptors disclosed herein are used in combination with a therapeutic agent, in particular a cytotoxic agent. Cytotoxic agents as used for the treatment of prostate cancer are known. Preferably such substances comprise taxol derivatives, 5-fluorouracil, cyclophosphamide, mitoxantrone, docetaxel, cabazitaxel and etoposide. The following drugs are approved for prostate cancer and preferably used: Abiraterone Acetate, Apalutamide, Bicalutamide, Cabazitaxel, Casodex (Bicalutamide), Degarelix, Docetaxel, Eligard (Leuprolide Acetate), Enzalutamide, Erleada (Apalutamide), Firmagon (Degarelix), Flutamide, Goserelin Acetate, Jevtana (Cabazitaxel), Leuprolide Acetate, Lupron Depot (Leuprolide Acetate), Mitoxantrone Hydrochloride, Nilandron (Nilutamide), Nilutamide, Provenge (Sipuleucel-T), Radium 223 Dichloride, Sipuleucel-T, Taxotere (Docetaxel), Xofigo (Radium 223 Dichloride), Xtandi (Enzalutamide), Zoladex (Goserelin Acetate), Zytiga (Abiraterone Acetate). It is understood that the chimeric antigen receptors of the present invention can also be used in combination with a medicament used to treat hormone sensitive forms of prostate cancer like for example Leuprolide Acetate.

The preferred embodiments of the present invention are further described and illustrated in the Figures and Examples of the present application. All aspects disclosed in the Figures or the examples, respectively, relate to the present invention unless expressly excluded. The

single features of the present invention as disclosed in the experimental part can be combined unless there are technical reasons which speak against such combination. In particular, the Figures show the results of the experiments as follows:

In the Figures and in the experiments the following abbreviations were used:

Abbreviation	Explanation
4-1BB	tumor necrosis factor receptor superfamily member 9
BLI	bioluminescence imaging
bw	body weight
C4-2	PSMA positive prostate cancer cell line
CAR	Chimeric Antigen Receptor
CAR28	anti-PSMA CAR with CD28 co-stimulatory domain
CAR41	anti-PSMA CAR with 4-1BB co-stimulatory domain
CD28	cluster of differentiation 28
CD3 ζ	CD3 zeta region
CD45RA	cluster of differentiation 45 isoform RA
CD62L	cluster of differentiation 62, L-selectin
CR	complete remission
DOC	docetaxel
DU 145	PSMA negative prostate cancer cell line
EFS	Short version of the elongation factor alpha gene promoter
ELISA	enzyme-linked immunosorbent assay
i.v	intravenously
i.p.	intraperitoneally
s.c.	subcutaneously
Gr.A	Granzyme A
Gr.B	Granzyme B.
IFN-g	Interferon-gamma
LTR	long terminal repeat of retroviral vector
PR	partial remission
R	R region of the retroviral long terminal repeat
scFv	single chain variable fragment
SD	standard deviation
Tcm	T cell central memory
Teff	T cell effector
Tem	T cell effector memory
Tn, scm	T cell naïve or T stem cell memory
U5	U5 region of the retroviral long terminal repeat

UT	untransduced T cell
WPRE	woodchuck hepatitis virus post-transcriptional regulatory element
XTT	sodium 3'-[1-(phenylaminocarbonyl)-3,4-tetrazolium]-bis(4-methoxy-6-nitro) benzene sulfonic acid hydrate
Δ U3	deletion in the U3 region in the retroviral long terminal repeat

Figure 1. Generation and quality assessment of PSMA targeting CAR T cells.

(A) Schematic of self-inactivating gamma-retroviral vectors to express 2nd generation anti-PSMA CARs. CAR expression is driven by an EFS promoter. The CAR consists of a single chain variable fragment D7 (derived from 3F/11 murine monoclonal antibody against PSMA), a hinge region (not shown), a transmembrane domain, a costimulatory domain either derived from 4-1BB (CAR41) or CD28 (CAR28), and an intracellular signaling domain derived from CD3 ζ chain. The hinge region, which is included in both CARs, is derived from human IgG and provides a physical spacer between the D7-scFv and the transmembrane domain for optimal target recognition.

(B) CAR expression. T cells were activated for 2-3 days with anti-CD2/3/28 antibodies prior to retrovirus transduction. Following viral transduction, T cells were expanded for 8-9 days before cells were harvested and stained with anti-human IgG antibody (CAR) and CD3 to evaluate the transduction efficiencies.

(C) Qualitative CAR T cell phenotype characterization with flow cytometric analysis. T cell subsets were evaluated by assessing the expression of CD62L and CD45RA, pre-gated on CAR-CD3⁺ for the untransduced T cells (UT) (not shown) or CAR+CD3⁺ for both types of CAR T cells (not shown).

(D) Qualitative assessment of CAR T cell phenotype. Shown are the average percentages of the different T cell subsets from at least three independent experiments. PSMA, prostate specific membrane antigen; scFv, single chain variable fragment; EFS, elongation factor 1 alpha short promoter; Tn/Tscm, T cell naïve or T stem cell memory; Tcm, T cell central memory; Tem, T cell effector memory; Teff, T cell effector.

This characterization of the CAR T cell phenotype confirms that the chosen CAR design enables the generation of high-quality of CAR T cell products with a minimal amount of terminally differentiated T cell subsets.

Figure 2. Cytotoxicity and cytokine release of PSMA-CAR T cells.

(A) Cytotoxicity profile of CAR T cells on PSMA positive C4-2 tumor cells. CAR T cells were co-cultured for 48 hours at different effector to target (E:T) ratios, with either antigen positive C4-2 tumor cells or PSMA negative cells (DU145) were used.

(B) Cytotoxicity of CAR T cells on PSMA-negative DU 145 tumor cells. CAR-T cells were co-cultured for 48 hours at different effector to target (E:T) ratios with DU145 cells.

Cytotoxicity was measured by an XTT ELISA based colorimetric assay (% cytotoxicity = 100 – % viability).

A comparison of the results as presented in Figure 2A and Figure 2B shows that the T cells transfected with the CAR, according to the invention, have a cytotoxic activity only with the cell line expressing the PSMA antigen since in panel A a PSMA expressing cell line (C4-2) was used. When the cell line does not express PSMA, as for example cell line DU 145, no cytotoxicity can be seen with the CAR T cells (Figure 2B). Furthermore, the CAR28 and CAR41 cells of this invention showed superior cytotoxicity as compared to previously published data (PMID 16204083, PMID: 18026115, PMID: 19773745, PMID: 25358763, PMID: 23242161, PMID: 4174378, PMID: 25279468): up to 90% of PSMA expressing cells were eliminated (Figure 2A), using a low E:T ratio of 1:1. The observed high in vitro cytotoxicity with a low E:T ratio of 1:1 is unique and was not described in any of the available PSMA-CARs so far, indicating the superior performance our CAR T cells based on the scFv D7.

(C) Quantification of pro-inflammatory cytokine release upon antigen stimulation. CAR T cells were stimulated with either PSMA-positive C4-2 cells or PSMA-negative DU 145 cells for 48 hours. Cytokines in supernatant were quantified by flow cytometry based cytokine-bead assay. Statistically significant differences are indicated by ***($P < 0.001$; $n=3$). UT; untransduced T cells, IFN- γ ; interferon gamma, Gr.A; Granzyme A, Gr.B; Granzyme B.

It can be seen from Figure 2C that pro-inflammatory cytokine release occurred only when CAR T cells were co-cultured with cell lines expressing PSMA (cell line: C4-2). CAR28 were activated to a higher extent than CAR41 cells.

Figure 3. CAR T cell phenotype and exhaustion profiles upon antigen specific stimulation.

(A) CAR T cell phenotype. CAR T cells were stimulated with PSMA-positive C4-2 tumor cells for 24 hours at a 1:1 effector-to-target (E:T) ratio before T cell phenotype profile was assessed based on the expression of CD62L and CD45RA. Cells were pre-gated on either CAR-CD3+ for UT control (not shown) or CAR+CD3+ for both types of CAR T cells (not shown).

(B) Quantitative assessment of CAR T cell phenotype. Shown are the average percentages of the different T cell subsets from three independent experiments. Statistical significant differences are indicated by ***($P < 0.001$) or *($P < 0.05$).

(C) T cell exhaustion profile. CAR T cells were stimulated with PSMA-positive tumor cells (C4-2) for 24 hours before T cell exhaustion profile was assessed by measuring the expression of CD223 (LAG-3) and CD279 (PD-1). Cells were pre-gated on CAR-CD3+ for UT control (not shown) or CAR+CD3+ for both types of CAR T cells (not shown).

(D) Quantitative assessment of CAR T cell exhaustion profiles. Shown are the average percentages of PD-1 or LAG-3 positive cells (three independent experiments). Statistically significant differences are indicated by ***($P < 0.001$). NS, not significant; UT, untransduced T cells; Tn/Tscm, T cell naïve or T stem cell memory; Tcm, T cell central memory; Tem, T cell effector memory; Teff, T cell effector; LAG-3, lymphocyte activation gene 3; PD-1, programmed cell death protein 1.

This set of experiments addressed the T cell phenotype and the exhaustion profiles of the CAR41 and CAR28 T cells upon antigen-specific stimulation. As seen in panels 3A and 3B, in contrast to CAR28 cells, a high percentage of CAR41 cells preserved an undifferentiated T cell phenotype upon antigen-specific stimulation. Furthermore, the CAR41 cells were less sensitive to exhaustion as compared to CAR28 T cells upon antigen-specific differentiation (Figures 3C and 3D).

Fig. 4: PSMA expression of the prostate cancer target cell line C4-2^{luc+}.

For in vivo bioluminescence imaging, PSMA-positive prostate cancer C4-2 cells were transduced with a lentiviral vector encoding firefly luciferase, a neomycin resistance gene and green-fluorescent protein (GFP). The transduced cell line was named C4-2^{luc+}.

(A) Binding of the anti-PSMA mAb 3/F11 to C4-2^{luc+} cells as determined by flow cytometry.

(B) Binding at a saturation concentration of 25 µg/ml 3/F11 to C4-2^{luc+} and PSMA-negative DU 145 cells as determined by flow cytometry.

Binding of the anti-PSMA mAb 3/F11, the parental mAb of the scFv D7, verified the PSMA expression on the surface of all C4-2^{luc+} prostate cancer target cells, while DU 145 prostate cancer control cells were shown to be PSMA negative.

Fig. 5: Bioluminescence of C4-2^{luc+} cells as determined by bioluminescence imaging (BLI) with the Living imaging system IVIS 200 (Xenogen VivoVision).

The luminescence of the C4-2^{luc+} target cells was tested using the In Vivo Imaging System IVIS 200 (Xenogen VivoVision) after incubation with luciferin. Compared to the non-transduced C4-2 cells, the C4-2^{luc+} cells showed bioluminescence, with an intensity (photons/sec/cm²) that was linear to the cell number. Thus, these cells were suitable for their use in the mouse tumor xenograft model with tumor detection by bioluminescence imaging (BLI). ROI, region of interest.

Fig. 6 Intratumoral CAR T cell therapy

(A) Schematic of intratumoral CAR T cell therapy. Tumor engraftment was established in animals by subcutaneous injection of 1.5×10^6 cells. When the growing solid C4-2^{luc+} tumors have reached 60-80 mm³ volume, CAR T cells were intratumorally injected with single doses of 5×10^6 CAR28 (n=5) or CAR41 T cells (n=5) at day 1 of treatment. Control mice were injected with the same number of UT cells (n=7) or left untreated (control, n=6). Tumors were visualized by bioluminescence imaging (BLI) until the end of treatment at day 22. D, day; i.t, intratumoral; s.c, subcutaneous.

(B) Antitumor activity of CAR28 and CAR41 T cells after intratumoral injection. Animals with subcutaneously growing solid C4-2^{luc+} tumors (60-80 mm³ volume) were intratumorally injected with single doses of 5×10^6 CAR28 (n=5) or CAR41 T cells (n=5) on day 1 of treatment. Control mice were injected with the same number of UT cells (n=7) or left untreated (control, n=6). Tumors were visualized by BLI until the end of treatment on day 22. Tumor volumes, represented as Mean $\pm$ SD were calculated from the BLI images using the formula $Vol = d^2 \times D / 2$, where d was the small diameter and D was the large diameter of the tumor. Statistical significant differences are indicated by ***p<0.001.

(C) Antitumor activity of intratumorally injected CAR28 and CAR41 T cells at the end of treatment (day 22). Tumor volumes were calculated from BLI images as described above. Tumor volumes are shown as Mean $\pm$ SD. Statistical significant differences are indicated by ***p<0.001. Since 1/6 mice of the control group developed an ulcerating tumor, this mouse had to be euthanized on day 15 and was not included into the statistical analysis of day 22.

(D) Representative bioluminescence images of mice intratumorally treated on day 1 of treatment with single injections of CAR28 or CAR41 T cells compared to untreated animals (control) or animals treated with UT T cells. Two mice representing animals with large or small tumors of each treatment group are shown.

Intratumoral treatment of mice bearing solid C4-2^{luc+} tumors of 60-80 mm³ volume with one injection of 5×10^6 CAR28 T cells on day 1 of treatment led to a significant inhibition of tumor growth and to 5/5 complete tumor remissions (CR) until day 15 (Figure 6B, C and D). One animal showed a recurrence of the tumor at day 22 with a tumor end volume of 0.55 mm³ (Figure 6D, mouse pictured). For all mice of the group a mean tumor volume of 0.11 ± 0.22 mm³ was calculated for day 22 (Figure 6B and C). Mice intratumorally treated with CAR41 T cells showed a different response. 1/5 CR and 1/5 partial remission (PR) were noted leading to a statistically significant inhibition of tumor growth with a mean tumor end volume of 397.3 ± 393.6 mm³ on day 22 (Figure 6B and C). In contrast, all mice of the UT and control groups showed tumor progression leading to tumor end volumes of 1469.4 ± 961.5 mm³ and 1289.1 ± 636.8 mm³, respectively (Figure 6B and C).

Fig. 7: Changes in body weight of intratumorally treated mice at the end of treatment (day 22).

According to animal protection guidelines, a decrease in body weight is an essential sign of toxicity during treatment. No animal showed a critical decrease of its initial body weight of more than 20%. This evidences that the intratumoral treatment was tolerated well without apparent signs of off-target toxicity.

Fig. 8: Intravenous CAR T cell therapy.

(A) Schematic of intravenous CAR T cell therapy. Tumor engraftment was established in animals by subcutaneous injection of 1.5×10^6 cells. When the growing solid C4-2^{luc+} tumors have reached 60-80 mm³ volume, CAR T cells were intravenously injected with single doses of 5×10^6 CAR28 (n=5) or CAR41 T cells (n=5) on day 1 of treatment. Control mice were injected with the same number of UT cells (n=7) or left untreated (control, n=6). Tumors were visualized via BLI until the end of treatment on day 22. D, day; i.v, intravenous; s.c, subcutaneous.

(B) Antitumor activity of CAR28 and CAR41 T cells after intravenous injection. Animals with subcutaneously growing solid C4-2^{luc+} tumors (60-80 mm³ volume) were intravenously injected with single doses of 5×10^6 CAR28 (n=5) or CAR41 T cells (n=5) at day 1 of treatment. Control mice were injected with the same number of untransduced T cells (UT cells) (n=5) or left untreated (control, n=6). Tumors were visualized via bioluminescence imaging until the end of treatment on day 22. Tumor volumes, represented as Mean $\pm$ SD were calculated from the BLI images using the formula $Vol = d^2 \times D / 2$, where d was the small diameter and D was the large diameter of the tumor.

(C) Antitumor activity of intravenously injected CAR28 and CAR41 T cells at the end of treatment (day 22). Tumor volumes were calculated from BLI images as described above. Tumor volumes are shown as Mean $\pm$ SD.

(D) Representative bioluminescence images of mice intravenously injected with single doses of CAR28 or CAR41 T cells. Two mice representing animals with large or small tumors of each treatment group are shown.

No inhibition of tumor growth could be measured after single intravenous injections of CAR28 or CAR41 T cells into mice bearing solid tumors with volumes of 60-80 mm³ (Figure 8B and C). As demonstrated in Figure 8C +D, animals of all treatment groups showed comparable tumor growth leading to tumor end volumes on day 22 of 1469.4 ± 961.4 mm³ (control), 1275.7 ± 544.5 mm³ (UT), 1427.3 ± 448.5 mm³ (CAR28), and 1529.3 ± 971.0 mm³ (CAR41).

Fig. 9: Combination therapy

(A) Schematic of combined chemotherapy with intravenous CAR T cell therapy. Tumor engraftment was established in animals by subcutaneous injection of 1.5x10⁶ cells. Chemotherapy had started at day 1 of the treatment for 3 days by intraperitoneal injection of docetaxel (DOC, 6 mg/kg bw). 48 hours after the last chemotherapy cycle, mice were injected intravenously with single doses of 5x10⁶ CAR28 (n=3) or CAR41 T cells (n=3). Control mice were injected with docetaxel alone (n=3) or left untreated (control, n=4). Tumors were visualized via BLI until the end of treatment at day 17. D, day; i.v, intravenous injection; i.p, intraperitoneal; s.c, subcutaneous.

(B) Antitumor activity of CAR28 and CAR41 T cells in combination with docetaxel chemotherapy. Mice with subcutaneously growing C4-2^{luc+} tumors (200–250 mm³) were intraperitoneally injected with docetaxel (DOC; 6 mg/kg bw) on days 1, 2, and 3 of treatment. 48 hours later mice were additionally injected with a single dose of 5x10⁶ CAR28 (n=3) or CAR41 T cells (n=3) intravenously. Tumor growth was monitored by BLI. Compared to the untreated control (n=4), DOC treatment led to a significant inhibition of tumor growth, which was enhanced by the addition of CAR28 or CAR41 T cells. 4/4 mice of the control group showed growing tumors during treatment, whereas in the DOC group 2/3 mice showed a PR. In the DOC + CAR28 group 1/3 animals showed a PR. In the DOC + CAR41 group 2/3 mice showed a CR and 1/3 mice a PR. A statistically significant difference between the DOC + CAR28 and DOC + CAR41 groups demonstrated the superior effects of the CAR41 T cells in this treatment scheme. Statistical significant differences are indicated by *p<0.05.

(C) Antitumor activity of CAR28 and CAR41 T cells in combination with docetaxel chemotherapy on day 17 of treatment. At the end of treatment on day 17 the mean tumor volume of mice of the control group was determined with 1817.5 ± 165.5 mm³. The DOC

group and the DOC + CAR28 group exhibited significantly lower volumes of $338.3 \pm 355.6 \text{ mm}^3$ and of $559.9 \pm 317.2 \text{ mm}^3$, respectively. Animals of the DOC + CAR41 group only had a mean tumor volume of $50.2 \pm 71.1 \text{ mm}^3$. Statistical significant differences are indicated by $*p < 0.05$.

(D) Representative bioluminescence images of mice pretreated with DOC and intravenously injected with single doses of CAR28 or CAR41 T cells. Two mice representing animals with large or small tumors of each treatment group are shown.

Taken together, pretreatment of tumors with docetaxel chemotherapy led to an antitumor activity of intravenously applicated CAR41 T cells.

Figure 10: Sequences

Figure 10 shows the amino acid sequence ("Frame1") of the antigen-binding construct D7 which is derived from mice. Moreover, the coding nucleic acid sequence and the complementary strand thereof are provided. The CDR H1-H3 and CDR L1-L3 nucleic acid and amino acid sequences are marked with grey arrows.

The amino acid sequence of the ScFv fragment of D7 (SEQ ID NO:1) is essential for the present invention insofar as it is the starting sequence for the humanization of the antigen binding fragment. The humanized sequences have a homology of at least 80% to SEQ ID NO:1. In a more preferred embodiment the sequences have a homology of at least 90% and more preferred at least 95% to SEQ ID NO:1 and even more preferred the homology is at least 98% to SEQ ID NO:1. It should be noted that the CDR regions which are shown in Figure 10 are conserved to a very high level which means that the CDR regions which are determined by the Kabat method have not more than three, preferably not more than one and preferably no amino acid exchange.

The sequences as disclosed in the present application are summarized as follows:

SEQ ID NO:	nucleic / amino acid	description	
1	amino acid	antigen binding fragment D7	Figure 10
2	amino acid	CDR-H1	Figure 10
3	amino acid	CDR-H2	Figure 10
4	amino acid	CDR-H3	Figure 10
5	amino acid	CDR-L1	Figure 10
6	amino acid	CDR-L2	Figure 10
7	amino acid	CDR-L3	Figure 10
8	coding sequence	D7	codon optimized for bacterial expresson
9	complementary strand	D7	codon optimized for bacterial expression
10	coding strand	CAR 28	Figure 12
11	coding strand	CAR 41	Figure 13
12	nucleic acid	coding strand D7	Figure 10
13	nucleic acid	complementary strand D7	Figure 10
14	amino acid sequence	} chimeric antigen receptor with fragment of CD 28	Figure 12
15	coding sequence		Figure 12
16	complementary strand		Figure 12
17	amino acid sequence	} chimeric antigen receptor with fragment of 4-1 BB	Figure 13
18	coding sequence		Figure 13
19	complementary strand		Figure 13
20	amino acid	D7 VH murine	Figure 14a
21	amino acid	hum D7 VH1	Figure 14a
22	amino acid	hum D7 VH2	Figure 14a
23	amino acid	hum D7 VH3	Figure 14a
24	amino acid	hum D7 VH4	Figure 14a

25	amino acid	hum D7 VH5	Figure 14a
26	amino acid	hum D7 VL1	Figure 14b
27	amino acid	hum D7 VL2	Figure 14b
28	amino acid	hum D7 VL3	Figure 14b
29	amino acid	hum D7 VL4	Figure 14b
30	amino acid	hum D7 VL5	Figure 14b
31	amino acid	D7 VL murine	Figure 14b
32	amino acid	D7 VH murine	Figure 11
33	amino acid	D7 VH hum 1	Figure 11
34	amino acid	D7 VH hum 2	Figure 11
35	amino acid	D7 VH hum 3	Figure 11
36	amino acid	D7 VH hum 4	Figure 11
37	amino acid	D7 VL murine	Figure 11
38	amino acid	D7 VL hum 1	Figure 11
39	amino acid	D7 VL hum 2	Figure 11
40	amino acid	D7 VL hum 3	Figure 11
41	amino acid	D7 VL hum 4	Figure 11

Figure 11: Potential mutations in humanized sequences

In Figure 11 the CDRs are shown. Usually the CDR-H1, CDR-H3 and CDR-L3 are without any amendments. It is possible, however, to replace one amino acid in CDR-L2, up to two amino acids in CDR-L1 and up to six amino acids in CDR-H3. The amino acids which can be replaced are designated as X1-X9 with the meaning shown in the legend to Figure 11.

Figure 12: Sequences of construct CAR28

Figures 12a-12d show the sequences of the construct designated as CAR-CD28.

Figure 13: Sequence of construct CAR41

The sequence of the construct CAR-4-1BB is shown in Figures 13a-13d.

It should be noted that in Figures 10-13 the sequence information is provided together with an information which function the relevant parts of the sequences have. Therefore, it is understood that the person skilled in the art derives the information in the best manner from the sequences provided. Sequences 10-13 disclose preferred embodiments of the invention.

Figure 14: Sequences of humanized ScFv derived from D7

(A) Shown are the murine sequence of the heavy chain of D7 and five humanized variants thereof whereby the locations of CDR-H1, CDR-H2 and CDR-H3 are shown according to the determination of Kabat and according to IMGT as well.

(B) Shown are the murine sequence of the light chain of D7 and of five humanized variants thereof, with CDR-L1, CDR-L2, and CDR-L3. It is understood that the heavy chains and the light chains can be combined with each other. It is for example possible to combine the heavy chain sequence of variant 1 (hum D7-VH1) with the light chain of variant 5 (hum D7-VL5).

Figure 15: Comparison of CAR constructs according to the invention with constructs wherein the PSMA antigen binding fragments were already described in the prior art

(A) Schematic of the self-inactivating retroviral vector constructs to express 2nd generation anti-PSMA CARs. CAR expression is driven by an EFS promoter. The anti-PSMA CARs contain either the single chain variable fragment (scFv) 3D8 or J591 (both described in the prior art), or D7 (derived from 3F/11 murine monoclonal antibody against PSMA), which is fused to an Fc IgG1 derived hinge region, a transmembrane (tm) domain, a CD28 derived costimulatory domain, and an intracellular signaling domain derived from CD3ζ chain. The hinge region provides a physical spacer between the scFv and the tm domains for optimal target recognition. The CD28 co-stimulatory domain contains amino acid exchanges that prevent LCK binding and enhance anti-tumor activity in the presence of inhibitory regulatory T cells (Tregs). All generated retroviral constructs have the same scaffold but differ only in the scFv fragments allowing for a side-by-side comparison of the CAR constructs with respect to activity and cytotoxicity.

(B) CAR expression in Jurkat cells were transduced with retroviral particles coding for the three different PSMA-CARs. Following viral transduction, Jurkat cells were expanded for 16 days before cells were harvested and stained with anti-human IgG antibody (CAR) to evaluate the transduction efficiencies and the CAR expression levels.

(C) Antigen-specific-activation profile, CAR expressing Jurkat cells were stimulated for 24 h at a 1:1 effector-to-target ratio with either PSMA positive, PD-L1 negative C4-2 tumor cells (PSMA+/PDL1-), or PSMA positive, PD-L1 positive LNCaP tumor cells (PSMA+/PDL1+). As a negative control, CAR cells were co-cultured with PSMA negative DU145 tumor cells (PSMA-). Following 24 h stimulation, cells were harvested and activation profile was assessed by evaluating the percentage of cells that are positive for the activation marker CD69.

The antigen-specific activation profiles were compared in D7-based CAR T Jurkat cells side-by-side with J591 and 3D8-based CAR T Jurkat cells. As indicated in Panel C, the D7-based CAR and the J591-based CAR were able to mediate massive activation of the Jurkat cells upon antigen-specific sensitization, as measured by upregulation of the activation marker CD69 in about -70% of cells. Activation was not affected by the presence of the inhibitory ligand PD-L1. On the other hand, the 3D8-based CAR T cells were only weakly activated (up to 20% of CD69-positive cells).

(D) CAR surface expression in primary T cells. T cells were activated for 2-3 days with anti-CD2/CD3/CD28 antibodies prior to retrovirus transduction. After an expansion for 6-9 days, cells were harvested and stained with anti-human IgG antibody (CAR) and CD3 to evaluate the transduction efficiencies and CAR expression levels.

(E-G) Cytotoxicity profile of CAR T cells were generated by transducing primary T cells after 3 days of activation with anti-CD3/CD28/CD2 antibodies. After an expansion for 8-9 days, the cytotoxicity profile was examined by co-culturing the CAR T cells for 48 hours at different effector-to-target (E:T) ratios, either C4-2 (PSMA+/PDL1-) tumor cells (E), LNCaP (PSMA+/PDL1+) tumor cells (F) or DU145 (PSMA-) tumor cells (G). Cytotoxicity was measured by an XTT ELISA based colorimetric assay (% cytotoxicity = 100% - % viability). Statistically significant differences are indicated by *($P < 0.05$), **($P < 0.01$), or ***($P < 0.001$).

UT, untransduced cells; PSMA, prostate specific membrane antigen; PD-L1, Programmed cell death ligand 1.

D7-based CAR T cells were compared side-by-side with J591-based CAR T cells. D7-based CAR T cells revealed a superior cytotoxicity profile as compared to J591-based CAR T cells on both tumor cell targets (E, F), as evidenced by the fact both tumor cell lines could be eliminated with lower effector-to-target ratios. Because of the weak activation profile (C) and the lack of efficient expression in T cells (D), 3D8-based CAR T cells were not further included in the comparison. In conclusion, both PSMA antigen-specific activation and cytotoxicity profile of the D7-based CAR T cells outperformed CAR T cells based on prior art CARs.

(H, I) Characterization of employed prostate cancer cell lines. The extent of PSMA target antigen expression (H) and PD-L1 (CD274) expression (I) was assessed on C4-2, LNCaP and DU145 prostate cell lines. For flow cytometric analysis, cells were either stained with 3/F11 antibody (anti-PSMA) or an anti-CD274 antibody. UT, untransduced T cells; PD-L1, Programmed cell death ligand 1; PSMA, prostate specific membrane antigen.

As shown in panel H, both C4-2 and LNCaP tumor cells express the PSMA antigen while the DU145 is negative for the PSMA antigen. Panel I shows that a large fraction of LNCaP and DU145 cells express PD-L1 while C4-2 cells were negative for PD-L1.

The results of the experiments shown in the Figures can be interpreted as follows:

In general, CARs are composed of an extracellular domain containing the antigen recognizing scFv, a hinge region, a transmembrane region, and one or more intracellular signaling domains that activate the T cell, including the CD3 ζ chain. In 2nd or 3rd generation CARs, co-stimulatory domains, usually derived from CD28, 4-1BB, OX40, CD27 and/or ICOS are included (**Figure 1A**). For the treatment of prostate cancer, many attempts had utilized CARs targeting PSMA epitopes and some of these strategies have already entered clinical trials (e.g. NCT01140373, NCT01929239). However, the potency of these CARs seems to be rather low both *in vitro* and *in vivo*. In particular, early studies based on 1st generation CARs based on the anti-PSMA scFv 3D8 or J591 (known as PzI), have shown low potency of the resulting CAR T cells, as indicated by the need of having to employ high effector to target (E:T) ratios up to 100:1 to eliminate the tumor cells *in vitro*. The *in vitro*

potency improved when 2nd or 3rd generation CARs based on either D2B or J591 derived scFvs were used. However, the potency of these CAR T cells remained low in xenotransplantation tumor mouse models, as indicated by the fact that these PSMA targeting CAR T cells were only able to suppress tumor growth but not to eliminate the tumors *in vivo*, although very high CAR T cell doses, sometimes up to 20×10^6 CAR T cells, or multiple infusions were applied (PMID: 16204083, PMID: 18026115, PMID: 19773745, PMID: 25358763, PMID: 23242161, PMID: 4174378, PMID: 25279468). In view of these mixed results with rather inefficient PSMA-CAR T cells, it was intended to generate and validate novel, more efficient PSMA targeting CARs based on the scFv D7. Two different 2nd generation PSMA-CARs were designed which either harbor a CD28 (CAR28) or 4-1BB (CAR41) derived co-stimulatory domain (**Figure 1**). The results demonstrate the high effectiveness of these newly developed D7-based PSMA-targeting CAR T cells both *in vitro* and *in vivo*. The manufactured CAR T cell products contained a high percentage of undifferentiated T cells, such as a naïve T cell, T stem cell memory and central memory T cell phenotypes (**Figure 1D**). These D7-based CAR T cells completely eliminated PSMA-positive tumor cells *in vitro* at a low effector to target ratio and released the expected cytokines upon specific antigen stimulation (**Figure 2A-C**).

CAR41 T cells maintained a more naïve phenotype and less exhaustion profile upon antigen specific stimulation as compared to CAR28 (**Figure 3**).

Importantly, the D7-based CAR T cells eliminated solid PSMA-positive tumors in a mouse tumor model upon intratumoral application (**Figure 6**). It can be seen that the control (untreated) and the control with untransduced (UT) T cells developed fast growing tumors. The two embodiments of the present invention (CAR28 T cells and CAR41 T cells), however, clearly blocked the tumor growth after an intratumoral single dose application and led to PR or CR. This proves that the concept works *in vivo*.

An intravenous single-dose injection of the CAR T cells of the present intervention did not cause growth inhibition of PSMA-positive solid tumors (**Figure 8**). However, after chemotherapy with docetaxel, a single dose of intravenously injected CAR41 T cells led to a complete remission of large tumors ($200\text{-}250\text{ mm}^3$) (**Figure 9**).

D7-based CAR T cells were compared side-by-side with J591 and 3D8-based CAR T cells (Figure 15). The antigen-specific activation profiles were compared in Jurkat cells

transduced with expression vectors coding for D7, J591 and 3D8-based CARs. While the D7-based CAR and the J591-based CAR were able to mediate massive activation of the Jurkat cells upon antigen-specific sensitization (Figure 15C), 3D8-CAR bearing cells were only weakly activated. Furthermore, upon transduction of primary T cells, D7-based CAR T cells revealed a superior cytotoxicity profile as compared to J591-based CAR T cells on two PSMA tumor cell lines (Figure 15E, 15F), as evidenced by the fact that both tumor cell lines were eliminated with lower effector-to-target ratios.

In summary, it has been demonstrated that the D7-based anti-PSMA CAR T cells have unexpected properties that are superior to previously published PSMA-targeting CAR T cells, particularly in view of their superior *in vitro* cytotoxicity and high *in vivo* antitumor activity. They are hence promising tools for the development of novel immunotherapies for the treatment of advanced prostate cancer.

The present invention relates therefore to chimeric antigen receptors for T cells which comprise an antigen-binding fragment which binds specifically to the PSMA antigen. The antigen-binding fragment comprises preferably a V_H and a V_L fragment which are connected with a suitable linker. Moreover, the chimeric antigen receptor comprises preferably a spacer element, a transmembrane fragment and a CDR3 ζ cytoplasmic domain. Furthermore, the chimeric antigen receptor preferably comprises a fragment from the CD28 (**Fig. 12**) and/or a 4-1BB (**Fig. 13**) cytoplasmic domain.

The chimeric antigen receptors of the present invention contain preferably at least three CDRs selected from the group consisting of CDR-H1, CDR-H2, CDR-H3, CDR-L1, CDR-L2 and CDR-L3 as shown in Figure 10. The CDRs are shown by grey arrows above the amino acid sequence and the relevant nucleic acid sequence coding. In a preferred embodiment the chimeric antigen receptor comprises at least three (CDR-H1, CDR-H3, CDR-L3), preferably four (CDR-H1, CDR-H3, CDR-L2, CDR-L3), and more preferred at least five (CDR-H1, CDR-H3, CDR-L1, CDR-L2, CDR-L3) of the CDRs as shown in Figure 10 and 11, respectively.

The chimeric antigen receptors are preferably present in a humanized format. Such a humanized format can be obtained by inserting the at least three, preferably four, more preferred five or six CDRs into a suitable human antigen-binding scaffold having a high homology to the murine scaffold as shown in **Fig. 11**.

Usually the genetic information coding for the chimeric antigen receptor is introduced with the help of a suitable vector into the target immune cells (e.g. T cell). Such a vector can preferably be a lentivirus vector or a retroviral vector or a transposon or a plasmid. Alternatively, the genetic information can be introduced into the genome of the T cell in a targeted fashion with the help of designer nuclease technology, such as the CRISPR/Cas technology or the TALEN technology, as described herein.

In a further aspect the present invention relates to an in vitro method of providing T cells comprising a chimeric antigen receptor as described herein. In the first step T cells are isolated from a donor preferably by leukapheresis methods. This results in a substantial enrichment of the T cells. The T cells are then genetically modified by transfection with a suitable vector or by transduction with a viral vector containing the genetic information for the chimeric antigen receptor, respectively. Such genetically modified T cells can then be isolated and amplified whereby those T cells which do not contain the desired genetic information can be separated from the modified T cells or at least reduced. The transfected T cells can then be applied to the patient to be treated.

Example 1

Preparation of CAR encoding retroviral particles

HEK293T cells were cultured at 37 °C in a humidified incubator with 5% CO₂ in DMEM (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 U/ml), streptomycin (100 mg/L) and 10 mM HEPES (Sigma-Aldrich). One day prior to transfection, cells were seeded in 10 cm dishes at cell density of 5x10⁶ cells/dish. 24 h later, cells were transfected using polyethylenimine (PEI: 0.1 mg PEI/ml, Polyscience Inc., USA). Per 10 cm dish 3 µg of a plasmid encoding the VSV-G envelope, 6 µg of a gag/pol encoding plasmid, and 10 µg of a vector plasmid coding for either CAR28 or CAR41 were used. 48 and 72 h post-transfection, supernatants containing viral vectors were collected and concentrated using ultracentrifugation (WX ultra series; Thermo Scientific: 25,000 rpm for 2 hours at 4°C). Concentrated vectors were suspended in 100 µl of cold PBS and kept at -80°C until used. Biological titers of the vector preparations were determined by transducing the Jurkat T cell line, followed by staining of the transduced cells with anti-human IgG to determine CAR positive cells.

Example 2***Generation of PSMA-targeting CAR T cells***

CAR T cells were generated from peripheral blood mononuclear cells (PBMCs). PBMCs were isolated using phase separation (Ficoll, Sigma-Aldrich) according to the manufacture's recommendation and then frozen in liquid nitrogen until used. For generation of CAR T cells, PBMCs were thawed and let to recover for 24 h in RPMI complete medium [RPMI 1640 medium (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany), penicillin (100 U/ml), streptomycin (100 mg/L) and 10 mM HEPES buffer (Sigma-Aldrich)]. Then, PBMCs were activated using anti-CD2/CD3/CD28 antibodies (Immunocult, StemCell Technologies) and cultured with RPMI complete medium supplemented with 100 U/ml of IL-2, 25 U/ml of IL-7 and 50 U/ml of IL-15 (all from Miltenyi Biotec) for 2 to 3 days before transduction with gamma-retroviral constructs encoding either CAR28 or CAR41 with MOIs ranging from 50-300. Transduced cells were cultured in wells coated with poly-D-lysine (PDL, Sigma-Aldrich) containing RPMI complete medium supplemented with 5 µg/ml of protamine sulfate (Sigma-Aldrich) and 1000 U/ml of IL-2, 25 U/ml of IL-7 and 50 U/ml of IL-15. After one day, medium was changed and cells were further expanded for 8-9 days in RPMI complete medium supplemented with 100 U/ml of IL-2, 25 U/ml of IL-7 and 50 U/ml of IL-15 before being frozen in liquid nitrogen until further use.

Example 3***Quality assessment of CAR T cells***

To monitor CAR and TCR expression by flow cytometry (FACS Canto II or Accuri, BD Biosciences), cells were stained with anti-human IgG-PE (Southern Biotech) and CD3-APC (Miltenyi Biotec), respectively. As shown in **Figure 1B**, up to 50% transduction efficiency was achieved for both CAR28 or CAR41. For quality assessment, CAR T cells were harvested and stained with anti-human CD62L-Bv421 (BD Biosciences), anti-human CD45RA-FITC (Biolegend), anti-human CD3-APC/H7 (BD Biosciences) and anti-human IgG-PE (CAR) (Southern Biotech) at the end of the expansion period (**Figure 1C, D**). The T cell phenotype was determined based on the expression of CD62L and CD45RA. Cells were pre-gated on CD3+/CAR- for untransduced (UT) T cells or on CD3+/CAR+ for both types of CAR T cells (**Figures 1C, D**). Both the transduction and expansion protocol did not induce T cell differentiation, as high percentages of undifferentiated cells with naïve T cell (Tn), T stem cell memory (Tscm) or central memory T cell (Tcm) phenotypes,

respectively, in combination with a low fraction of terminally differentiated effector T cells (Teff) were present (**Figures 1C, D**), indicating that the protocol allowed for the generation of high quality CAR T cell products.

Example 4

In vitro cytotoxicity of manufactured CAR T cells

The cytotoxic potential of the manufactured CAR T cells was determined by assessing cell viability using the XTT assay, as previously described. CAR T cells were co-cultured with either PSMA-positive C4-2 tumor cells or antigen-negative tumor control cells (Du145) in 96 well plates for 48 h at different effector to target ratios in a final volume of 200 µl/well of RPMI complete medium without any cytokines. To determine cell viability as a function of metabolic activity, 100 µl/well of medium was removed and replaced with 100 µl/well of XTT solution (Sigma-Aldrich) and cells incubated at 37°C. Colorimetric changes were quantified using an ELISA reader (Infinite F50, Tecan) at 450 nm. Cytotoxicity is indicated as the percentage of dead cells, which equals 100% minus the percentage of viable cells. Viability was calculated according to the equation $[\text{OD}_{\text{E+T}} - \text{OD}_{\text{E only}}]/[\text{OD}_{\text{T only}} - \text{OD}_{\text{medium only}}]$ (E, effector cells = CAR T cells; T, target cells = tumor cells). As shown in **Figure 2A**, CAR28 T cells eliminated ~90% of target cells at an effector to target (E:T) ratio of 1:4, while CAR41 T cells demonstrated comparable activity at an E:T ratio of 1:1. Both types of CAR T cells revealed some minimal alloreactivity when co-cultured with PSMA-negative tumor cells (DU 145) (**Figure 2B**). Together, the results demonstrate that both types of D7-based CAR T cells eliminate PSMA-positive target cells with high efficiency and specificity.

Example 5

Cytokine release by activated CAR T cells

CAR T cells were co-cultured with either PSMA-positive C4-2 tumor cells or antigen negative DU 145 tumor cells for 48 h at an effector to target ratio of 1:1 in a final volume of 200 µl/well of RPMI complete medium without any cytokines. To evaluate the cytokines that were released from the CAR T cells during this time, supernatants were collected and evaluated by a multiplexed bead-based immunoassay (cytometric bead array, CBA assay, BD Biosciences) according to the manufacturer's recommendations. Three analytes, interferon gamma (IFN-γ), granzyme A (Gr.A) and granzyme B (Gr.B), were determined (**Figure 2C**).

Both types of the D7-based CAR T cells secreted the measured pro-inflammatory cytokines upon antigen stimulation, although CAR41 T cells released significantly less cytokines than CAR28 T cells.

Example 6

Cell differentiation and exhaustion upon antigen stimulation

CAR T cells were stimulated with PSMA-positive C4-2 tumor cells for 24 h at an effector to target ratio of 1:1, before cells were harvested and stained for T cell phenotype and exhaustion profiles. Assessment of the CAR T cell phenotype upon antigen stimulation revealed that CAR41 cells were less prone to differentiate as compared to CAR28 cells, as indicated by the presence of a significantly higher number of undifferentiated T cell subtypes, such as T_n, T_{scm} and T_{cm} (**Figure 3A, B**).

To monitor CAR T cell exhaustion upon specific antigen stimulation, CAR T cells were stimulated with PSMA-positive tumor cells (C4-2) for 24 h, before cells were harvested and stained with anti-human CD279-FITC (PD-1, BD Biosciences), anti-human CD223-eFluor710 (LAG-3, BD Biosciences), anti-human CD3-APC/H7 (BD Biosciences) and anti-human IgG-PE (Southern Biotech). The exhaustion profile was determined by flow cytometry (FACS Canto II) based on the expression of CD279 (PD-1) and CD223 (LAG-3). Cells were pre-gated either on CD3⁺/CAR⁻ for UT T cells or CD3⁺/CAR⁺ for both types of CARs. Yet again, CAR41 cells displayed a different phenotype than CAR28 cells. The significantly reduced number of LAG-3 positive CAR41 T cells is indicative of a less exhausted T cell phenotype (**Figure 3C, D**).

Example 7

PSMA expression of the prostate cancer target cells C4-2^{luc+}

The PSMA expressing, androgen-independent prostate cancer cell line C4-2 was grown in RPMI 1640 medium (Gibco, Invitrogen, Karlsruhe, Germany) supplemented with penicillin (100 U/ml), streptomycin (100 mg/L) and 10% fetal calf serum (FCS, Biochrom, Berlin, Germany) at 37 °C in a humidified atmosphere of 5% CO₂. For *in vivo* bioluminescence imaging, C4-2 cells were transduced with a lentiviral vector encoding firefly luciferase, a neomycin resistance gene, and green-fluorescent protein (GFP). The cells were named C4-2^{luc+} cells and selected with 2mg/ml of the neomycin analog Geneticin (G-418 Sulfate) as previously described. C4-2^{luc+} cells were tested for PSMA expression by flow cytometry as described previously. In brief, 2x10⁵ C4-2^{luc+} cells/well in PBS with 3% FCS and 0.1%

NaN₃ were incubated with different concentrations of the anti-PSMA mAb 3/F11, the parental mAb of the scFv D7, for 1 h on ice. After washing, cells were incubated with 25 µl goat anti-mouse Ig-RPE (Becton-Dickinson, Mountain View, CA) for 40 min on ice. Cells were then washed repeatedly and suspended in 200 µl PBS containing 1 µg/ml propidium iodide, 3% FCS and 0.1% NaN₃. Analysis of stained cells was performed using a FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences, Heidelberg, Germany).

As shown in **Figure 4A**, the mAb 3/F11 bound with a binding constant (kd), defined as the half-maximal saturation concentration, of 0.96 µg/ml (about 6.3 nM) to the C4-2^{luc+} cells. This proved the high expression of PSMA on this cell line. C4-2^{luc+} cells were shown to be PSMA positive at saturation concentrations, while DU 145 prostate cancer control cells were shown to be PSMA negative (**Figure 4B**).

Example 8

Bioluminescence of C4-2^{luc+} cells

Bioluminescence of the C4-2^{luc+} cells was tested with help of the *in vivo* imaging system IVIS 200 (Xenogen VivoVision). For this, cells were seeded in different numbers into 96-well plates (Nunc Delta Surface, Thermo Fisher Scientific, Roskilde, Denmark) and incubated with 40 µl luciferin/well (BioSynth AG, Staad, Switzerland) as a substrate. Luminescence was analyzed using the software Living Image 3.0 within 10-30 min (Caliper Lifesciences).

Bioluminescence Imaging (BLI) showed that there was linearity between the number of C4-2^{luc+} cells and bioluminescence (quantified as region of interest (ROI)). No luminescence signal could be detected with untransduced C4-2 cells and background staining was determined with ROI of max. 2.362×10^6 photons/sec/cm² (**Figure 5**).

Example 9

Treatment of C4-2^{luc+} tumor xenografts by intratumoral injection of CAR T cells

5-6 week old male SCID CB17/lcr-Prkdc scid/Crl mice (20-25 g, Janvier Labs, St Berthevin Cedex, France) were injected with 1.5×10^6 C4-2^{luc+} cells in PBS mixed with 50% Matrigel (Collaborative Biomedical Products, Chicago, IL) s.c. into the right flank. Tumor growth was monitored by BLI. For this, 200 µl of luciferin (BioSynth AG, Staad, Switzerland) were injected i.p. into the animals and BLI was done 10-30 min after injection under anesthesia

using the Living imaging system IVIS 200 (Xenogen VivoVision). Tumor volumes were calculated from BLI pictures using the software Living Image 3.0 and the formula $\text{Volume} = d^2 \times D / 2$, where D was the large diameter and d the small diameter of the tumor. When tumors reached volumes of about 60-80 mm³, mice were injected intratumorally with only one dose of 5×10^6 CAR28 (n=5) or CAR41 T cells (n=5), respectively (day 1 of treatment) (**Figure 6A**). As controls, mice were injected with un-transduced T cells (UT, n=7) or left untreated (control, n=6). Tumor growth was monitored via BLI on days 1, 3, 8, 15, and 22 of treatment. Control groups (UT and untreated) showed a fast tumor growth. A reduced tumor growth was reached with the CAR41 T cells. A marked tumor regression was found in mice treated with CAR28 T cells (**Figure 6B, C, and D**). Until end of treatment at day 22, all animals of the control groups showed growing tumors. According to the animal protection guidelines, one mouse of the untreated group had to be killed due to an ulcerating tumor at day 15.

At day 22, mean tumor end volumes of 1469.4 ± 961.5 mm³ (untreated control) and 1289.1 ± 636.8 mm³ (UT) were reached, respectively. In contrast, 5/5 mice treated with CAR28 T cells showed a complete tumor remission (CR) from day 8 on and 4 mice remained tumor free until the end of the experiment. In the remaining animal a small tumor was detected at day 22 and verified by histological examination with a volume of 0.55 mm³. For all mice treated with CAR28, a mean tumor volume of 0.11 ± 0.22 mm³ was calculated for day 22, which was statistically significant compared to the control groups (*p < 0.05) (**Figure 6B and 6D**). Mice injected intratumorally with CAR41 T cells showed a different response to the treatment. One of the five animals treated showed a CR (**Figure 6B and 6D**). Another animal showed a PR, whereas the others had tumor progression until day 22 of treatment. In summary, a mean tumor volume in this group of 397.7 ± 393.6 mm³ was determined (**Figure 6C**).

Example 10

Adverse side effects

According to the animal protection guidelines, mice were observed for apparent signs of toxicity during treatment (loss of weight and appetite, changes in pelage, fever, tension, apathy, aggression, respiratory disorders, paralyses, death) and termination criteria were defined as follows: significantly reduced food intake, decrease of the initial body weight >20%, spasms, paralysis, abnormal breathing disorders, apathy, aggressiveness as a sign of severe pain, tumor diameter >20 mm, ulcerating tumor. With exception of one mouse of

the untreated control group, which had to be killed at day 15 because of an ulcerating tumor, no mouse showed apparent signs of toxicity and no animal reached one or more determination criteria. **Figure 7** demonstrates that no animal showed a critical decrease of its initial body weight of >20%. Taken together, all animals have tolerated the intratumoral treatment with the anti-PSMA CAR T cells well without apparent signs of unspecific toxicity.

Example 11

Treatment of C4-2^{luc+} tumor xenografts by intravenous injection of CAR T cells

Mice with C4-2^{luc+} tumor xenografts were intravenously injected with only one dose of 5×10^6 CAR28 (n=5), CAR41 (n=5), or UT T cells (n=5) in 100 μ l of PBS, respectively (day 1 of treatment) (**Figure 8A**). Control mice (n=6) remained untreated. Tumor growth was monitored as described above. Compared to the control (tumor end volume: 1469.4 ± 961.5 mm³) and the UT group (tumor end volume: 1275.7 ± 544.5 mm³), mice injected with CAR28 T cells showed a similar tumor growth with a mean tumor volume of 1427.3 ± 448.5 mm³ at the end of treatment at day 22 (**Figure 8B and 8C**). For animals treated with the CAR41 T cells a mean tumor volume of 1529.3 ± 971.0 mm³ was determined (**Figure 8C**). Tumor growth was not controlled in animals treated either with CAR41 or CAR28 intravenously (**Figure 8B, C, and D**).

In summary, intratumoral application of PSMA CAR T cells, both CAR28 and CAR41, completely eliminated tumors *in vivo* (**Figure 6**). In contrast, intravenous injection of the CAR T cells did not lead to any antitumor activity (**Figure 8**), suggesting that the intratumoral injection allowed the CAR T cells to bypass the TME, while upon intravenous injection the CAR T cells were likely inhibited by the TME.

Example 12

Treatment of C4-2^{luc+} tumor xenografts by combination of chemotherapy and intravenous injection of CAR T cells

Mice with large C4-2^{luc+} tumor xenografts (200-250 mm³) were intraperitoneally injected with 3 daily cycles of docetaxel (DOC, 6 mg/kg bw on days 1, 2, and 3 of treatment) (**Figure 9A**). 48 hours after the last cycle, mice were intravenously injected with only one dose of 5×10^6 CAR28 (n=3) CAR41 T cells (n=3), or PBS (n=3) respectively (**Figure 9A**). Control animals (n=4) remained completely untreated. Tumor growth was monitored by BLI as described above. Compared to the control group, mice injected with DOC alone and

with DOC + CAR28 showed an inhibition of tumor growth (**Figure 9B**). Compared to the control group ($1817.5 \pm 165.5 \text{ mm}^3$) tumor end volumes of $338.2 \pm 355.6 \text{ mm}^3$ for mice treated with DOC alone and of $599.9 \pm 317.2 \text{ mm}^3$ for mice treated with DOC + CAR28 were reached on day 17 (**Figure 9C**). In contrast, 2/3 animals treated with the DOC + CAR41 combination therapy showed CR and 1/3 mice a PR (**Figure 9B**). A mean tumor volume of $50.2 \pm 71.0 \text{ mm}^3$ was determined (**Figure 9C**).

These results indicate that the D7-CAR T cells are effective in eliminating large tumors upon a single dose administration of CAR T cells in combination with chemotherapeutic agents, such as docetaxel (**Figure 9D**).

Example 13

Humanization of murine sequences

In order to avoid later potential disadvantageous effects like antibodies generated in human patients against the murine part of the antigen binding fragment, the antigen binding fragment derived from the construct D7 was humanized.

In the course of humanizing the sequences the anti-PSMA binding sequence derived from the construct D7 was compared with the sequences of human stem cells. A sequence having a very high homology was selected and the area wherein the CDR binding sequences are located has been determined. For the determination of the CDR sequences several methods are known. The most preferred methods are the method according to Kabat and the IMGT method. IMGT, the International Immunogenetics database, is a highly qualified integrated information system specializing in immunoglobulin molecules. Since the methods according to Kabat and IMGT provide somewhat different results, the sequences of different variants (hum D7 VH1-5 and hum D7 VL1-5) are shown in Figure 14 A and B. The relevant CDR regions according to Kabat and to IMGT are shown.

It is important to note that even by using the two different methods the sequence of CDRH3 having the amino acid sequence ARDGNFPYYAMDS (SEQ ID NO:11) and in CDRL3 having the sequence SQSTHVPT (SEQ ID NO:12) are identical according to both determination methods. It is therefore assumed that those two CDRs, namely CDRH3 and CDRL3 are important for the proper function of the construct. The humanized sequences as shown in Fig. 14a and b correspond to SEQ ID NO:13 to 23.

Example 14

The biological activity of a preferred construct according to the present invention has been compared with a construct wherein the PSMA binding fragment is derived from the antibody J591. Furthermore, it was compared with a similar construct wherein the antigen binding construct was derived from another antibody (3D8).

The experiments and the results obtained therefrom are depicted in Figures 15A-15I.

Figure 15A is a schematic representation of different anti-PSMA-CAR28 constructs which are identical except the PSMA binding fragment. In construct CAR28 (D7) the fragment according to the invention was used. In CAR28 (J591) the PSMA fragment was derived from the antibody J591 and in CAR28 (3D8) the PSMA fragment was derived from a PSMA binding antibody (3D8).

The constructs were introduced into Jurkat T-cell lines and the expression of the constructs was measured. In Figure 15B the control is designated as UT (untransduced). Figure 15B shows that all CAR constructs measured in flow cytometry are properly expressed in the Jurkat T-cell line except the control, which is designated as UT (untransduced).

In Figure 15C the antigen-specific activation profile of the transduced JURKAT cells is measured by monitoring the expression of the activation marker upon antigen stimulation. As opposed to D7 and J591 based CARs the 3D8 based CAR mediated only weak activation of the transduced Jurkat cells upon expansion to PSMA expressed tumor cells.

Figure 15D shows the CAR expression in primary T-cells. It can be seen that the construct according to the invention CAR28 (D7) is better expressed (42%) compared with CAR28 (J591) (30%). The expression of the construct wherein the PSMA binding fragment is derived from 3D8 is very low (4%). A construct which is not expressed to a sufficient extent in primary T-cells is not suitable for later therapeutic use.

The cytotoxicity profiles of the CAR28 T-cells on indicator cells, namely C4-2 (PSMA+/PDL1-); LNCap (PSMA+/PDL1+) and DU145 (PSMA-/PDL1+) is shown in in

Figure 15E-15G. To assess the cytotoxicity profile of the CAR T cells different ratios of effector (CAR28 T-cells):Target cells (different tumor cells, namely C4-2, LNCaP and DU145) have been determined. The construct according to the invention is superior to constructs having the antigen binding fragment derived from the construct J591, as can be seen from **Figure 15E** and **Figure 15F**. At lower effector:target ratios the construct according to the invention is always superior than the comparative construct. The effector target ratio is a very important aspect for the clinical use since very high effector:target ratios cannot be provided in real life. The lower the effector:target ratio is, the better the construct is.

Figure 15H and **Figure 15I** show the PSMA and PD-L1 expression on the prostate cancer cell lines LNCaP, C4-2 and DU145.

Example 15

In an additional experiment it could be shown that a combination of chemotherapy together with CAR therapy is superior to chemotherapy alone.

The experiments are shown in **Figure 16A** and **Figure 16B**. **Figure 16A** is a schematic of combination therapy of mice bearing subcutaneous C4-2^{luc} xenografts with docetaxel (DOC) plus CAR T cells. Mice with 150-200 mm³ tumors were injected with 2 doses of 6 mg/kg bw DOC on days 1 and 2 followed by i.v. injection of CAR28 or CAR41 T cells on day 8. Tumor growth was monitored by palpation and BLI until day 22.

The results of the experiments are shown in **Figure 16B**: in vivo antitumor activity of CAR28 and CAR41 T cells in combination with DOC in mice bearing subcutaneous C4-2^{luc} xenografts. Statistically significant differences in tumor volume between DOC + CAR28 treated animals and untreated control were determined on days 15, 17, and 22 of treatment. Statistically significant differences in tumor volume between DOC + CAR28 treated animals and mice, which were only treated with DOC, were determined on days 17 and 22 of treatment. Moreover, tumor volume of animals treated with DOC + CAR41 was significantly smaller than that of the control group on day 22. This proves that combination treatment was superior to chemotherapy alone. Unpaired t-test, *p<0.05.

From the experiments it can be clearly seen that there is a synergistic effect of the CAR28 and CAR 41 constructs according to the invention together with a chemotherapeutic agent whereby the effect has been exemplified with docetaxel.

Claims

1. Chimeric antigen receptor comprising an antigen-binding fragment which binds specifically to PSMA antigen, whereby the antigen receptor against PSMA is derived from the single-chain variable fragment D7, a transmembrane domain, in particular a CD3 ζ cytoplasmatic domain and an intracellular signaling domain, in particular a CD28 and/or a 4-1BB cytoplasmatic domain.
2. Chimeric antigen receptor according to claim 1 characterized in that it contains at least three CDRs selected from SEQ ID NO:2, SEQ ID NO:4 and SEQ ID NO:7 or in that it contains at least four CDRs selected from SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:6 and SEQ ID NO:7, or in that it contains at least five CDRs selected from SEQ ID NO:2, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7, or in that it contains at least six CDRs selected from the group consisting of SEQ ID NO:2, SEQ ID NO:3, SEQ ID NO:4, SEQ ID NO:5, SEQ ID NO:6 and SEQ ID NO:7.
3. Chimeric antigen receptor according to claim 1 characterized in that the antigen-binding fragment is humanized.
4. Chimeric antigen receptor according to claim 3 characterized in that it is derived from the single-chain variable fragment D7.
5. Chimeric antigen receptor according to claim 3 or 4 characterized in that it comprises the amino acid sequence: ARDGNFPYYAMDSW.
6. Chimeric antigen receptor according to claim 3 or 4 characterized in that it comprises the amino acid sequence: SQSTHVPT.
7. Nucleic acid coding for a chimeric antigen receptor according to any of claims 1 to 6 characterized in that it contains the nucleic acid coding for the chimeric antigen receptor according to any of claims to 6.

8. Vector coding for a chimeric antigen receptor according to any of claims 1-6 characterized in that it contains the nucleic acid coding for the chimeric antigen receptor according to any of claims 1-7.
9. Vector according to claim 8 characterized in that it comprises SEQ ID NO:10 or SEQ ID NO:11 or a sequence which is identical therewith at a degree of at least 95%.
10. *In vitro* method of providing immune cells, in particular T cells comprising a chimeric antigen receptor according to any of claims 1-6 comprising
isolating immune cells, in particular T cells from a donor by leukapheresis;
transfecting/transducing the immune cells, in particular T cells with a vector according to claim 8 coding for a chimeric antigen receptor;
isolating and amplifying the transfected/transduced T cells.
11. Immune cell, in particular T cell, characterized in that it contains the genetic information coding for a chimeric antigen receptor according to any of claims 1-7.
12. Chimeric antigen receptor according to any of claims 1-6 or a nucleic acid according to claim 7 or a vector according to claims 8 or 9 for use in the treatment of prostate cancer and/or prostate-derived tumors.
13. Chimeric antigen receptor according to any of claims 1 to 6 and/or nucleic acid according to claim 7 and/or vector according to claim 8 or 9 for use in the treatment of solid tumor expressing PSMA wherein the agent having anti-tumor activity is introduced into the solid tumor.
14. Chimeric antigen receptor for use according to any of claims 12-13 characterized in that it is combined with the administration of chemotherapeutically active agents whereby the chemotherapeutically active agents are selected from cytotoxic substances comprising taxol derivatives, 5-fluorouracil, cyclophosphamide, mitoxanthrone, docetaxel and/or capecitaxel.
15. Chimeric antigen receptor for use according to any of claims 12-14 characterized in that it is combined with the administration of a biopharmaceutical, comprising proteins, antibodies, vaccines, blood, blood components, allergenics, recombinant therapeutic

proteins, gene therapies, somatic cells, tissues, and cell therapies, in particular protein kinase inhibitors, enzyme inhibitors, anti-genomic therapeutics.

Fig.1

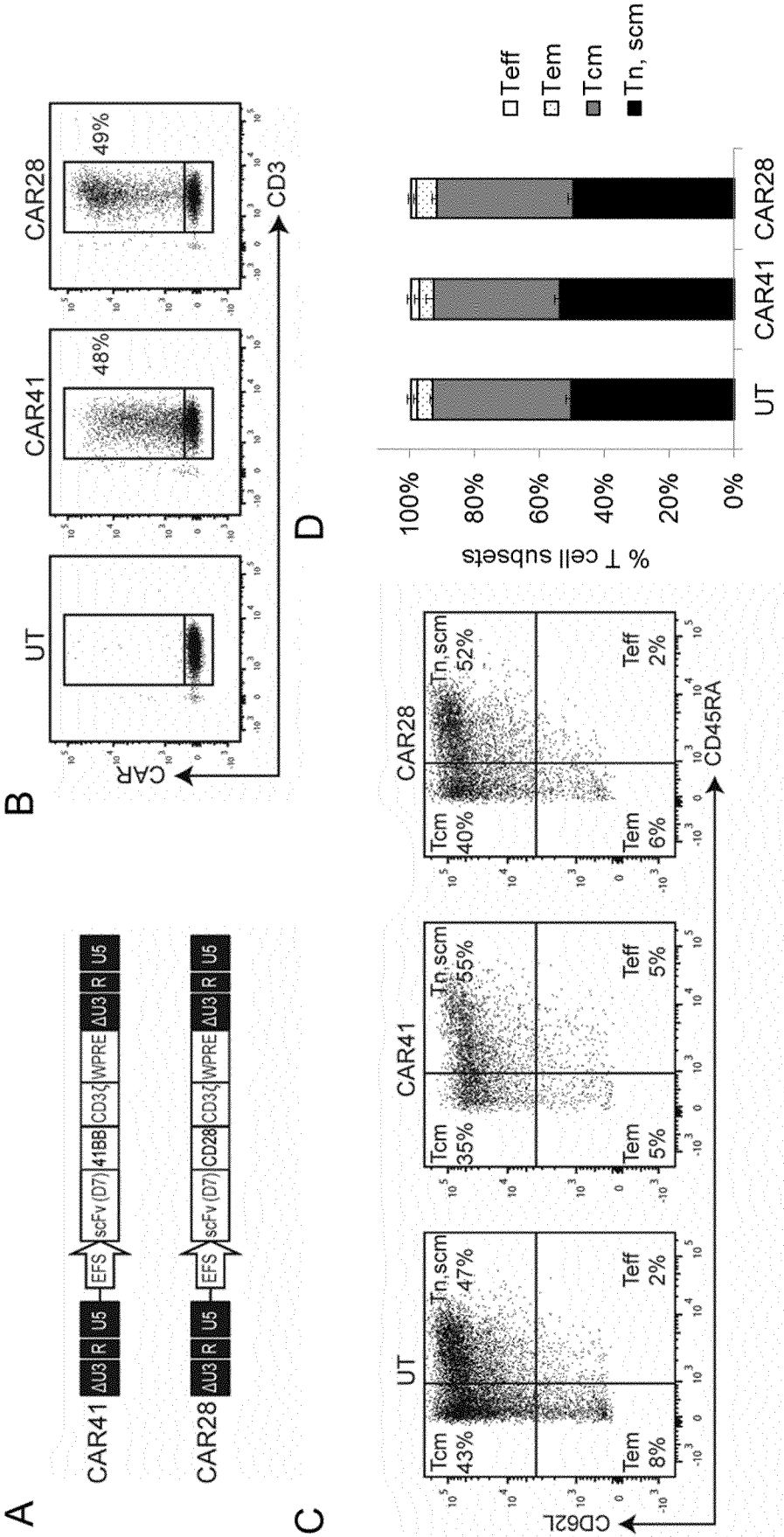


Fig.2

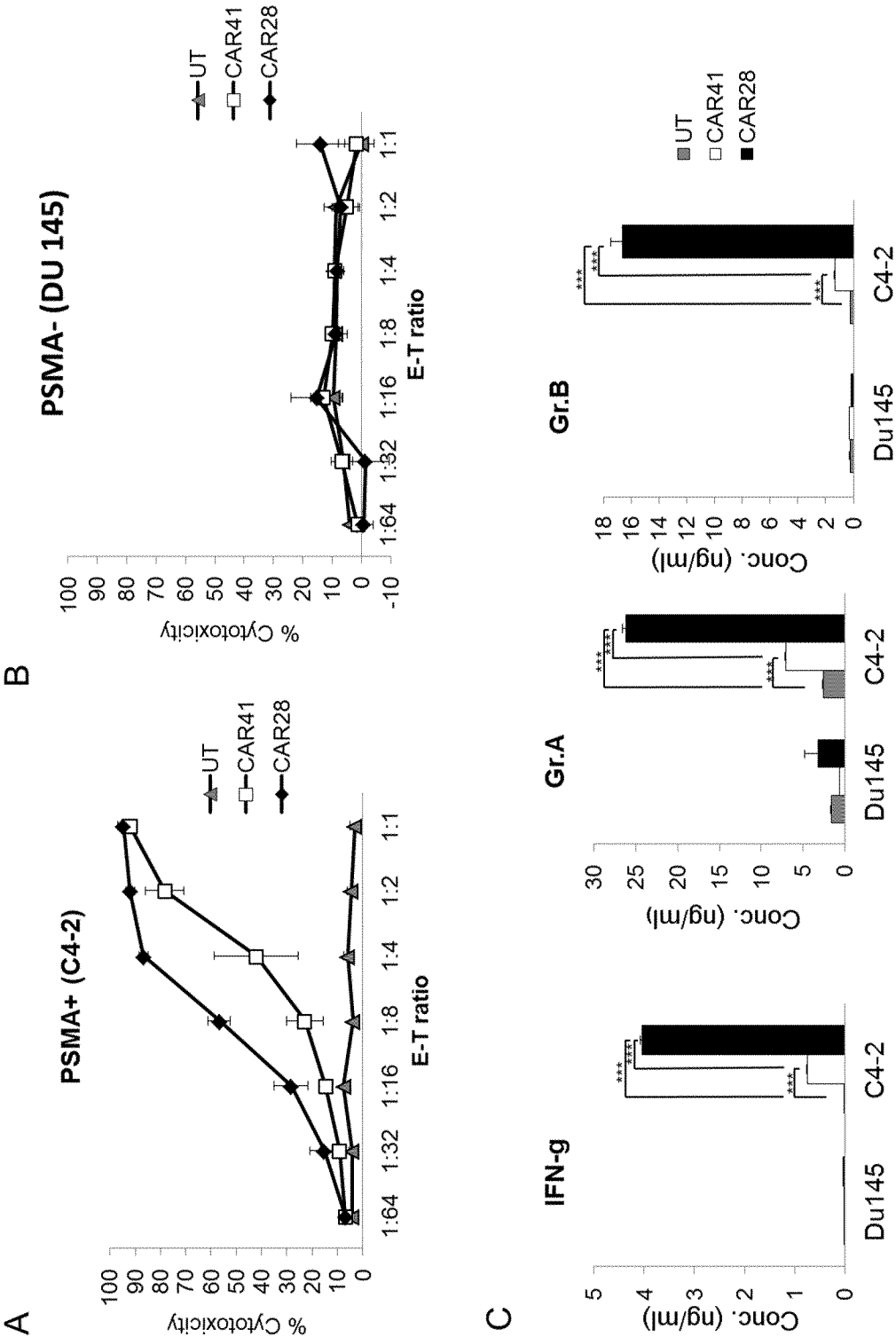


Fig.3

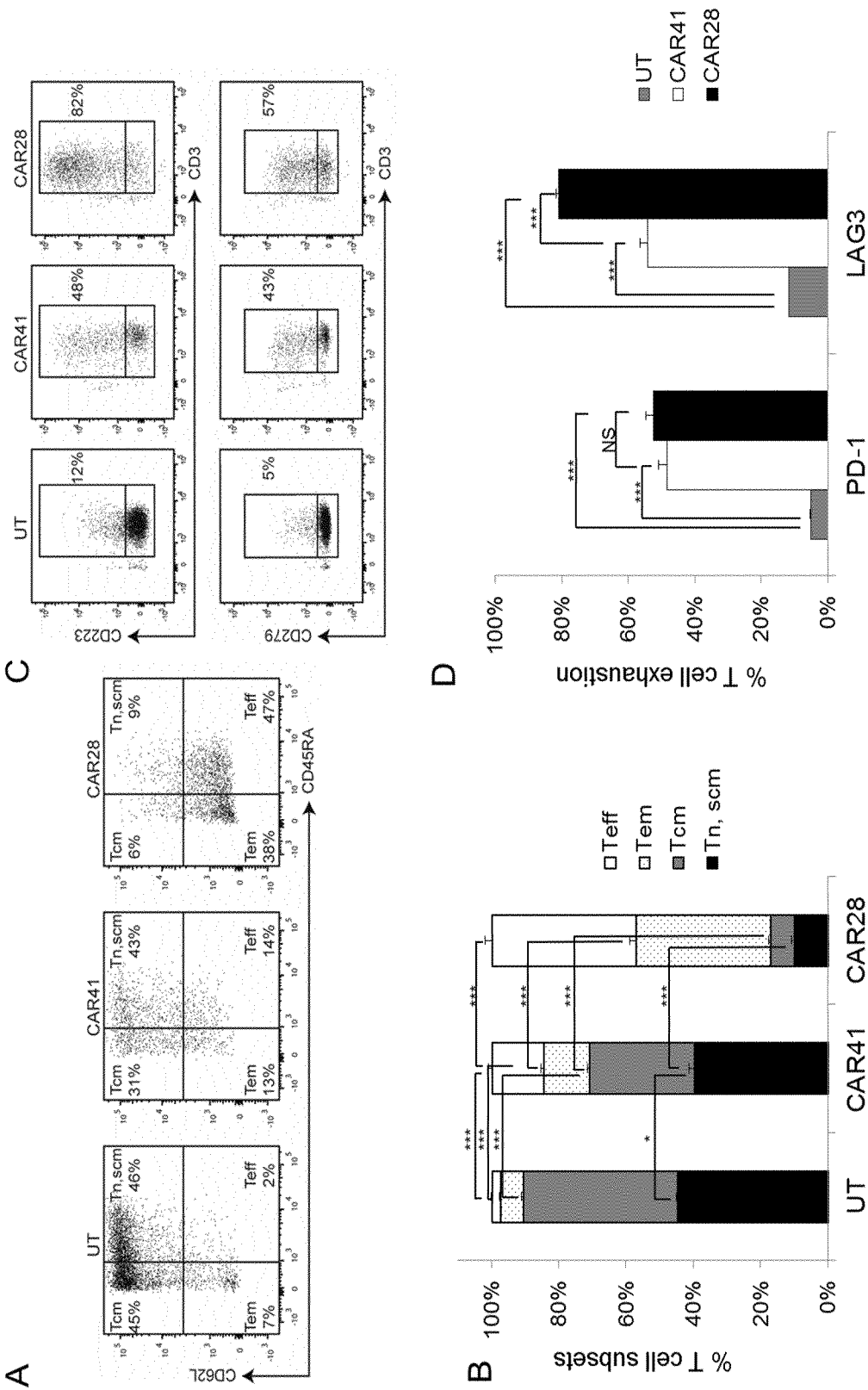
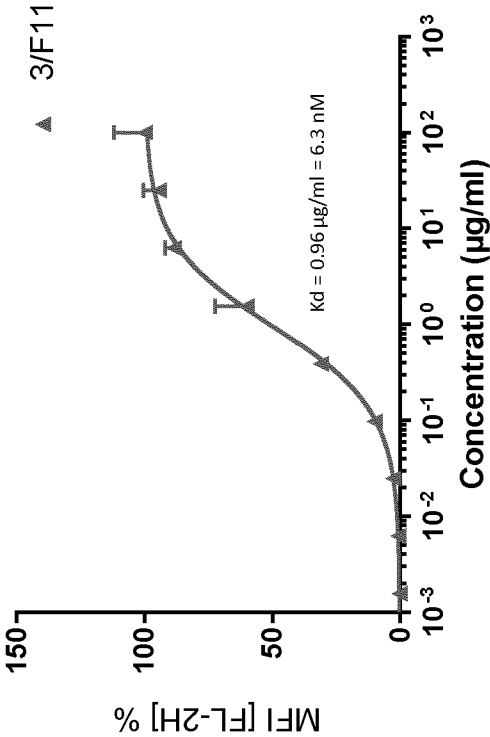


Fig.4

A



B

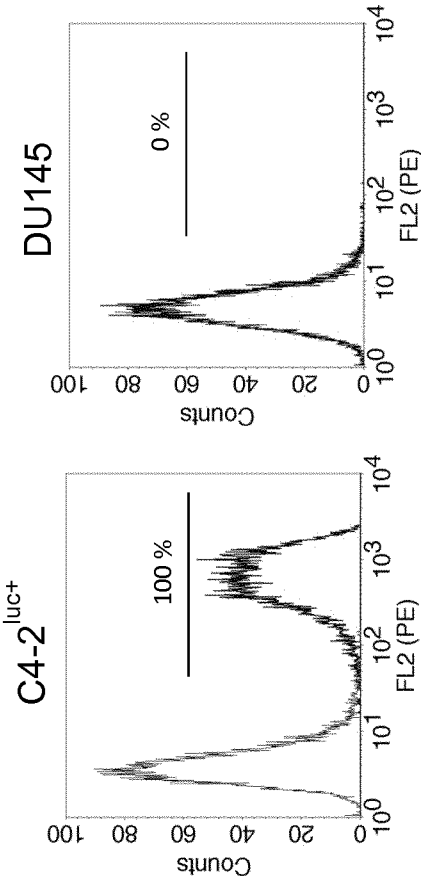


Fig.5

cell number ($\times 10^5$ /well):

C4-2 cells

C4-2^{luc+} cells

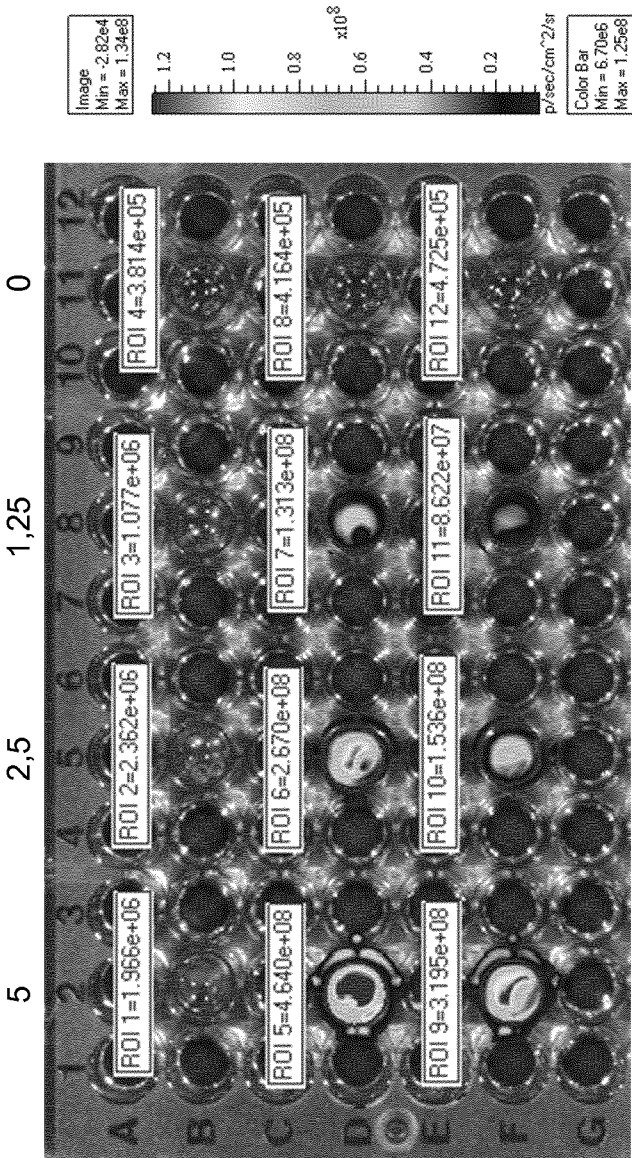
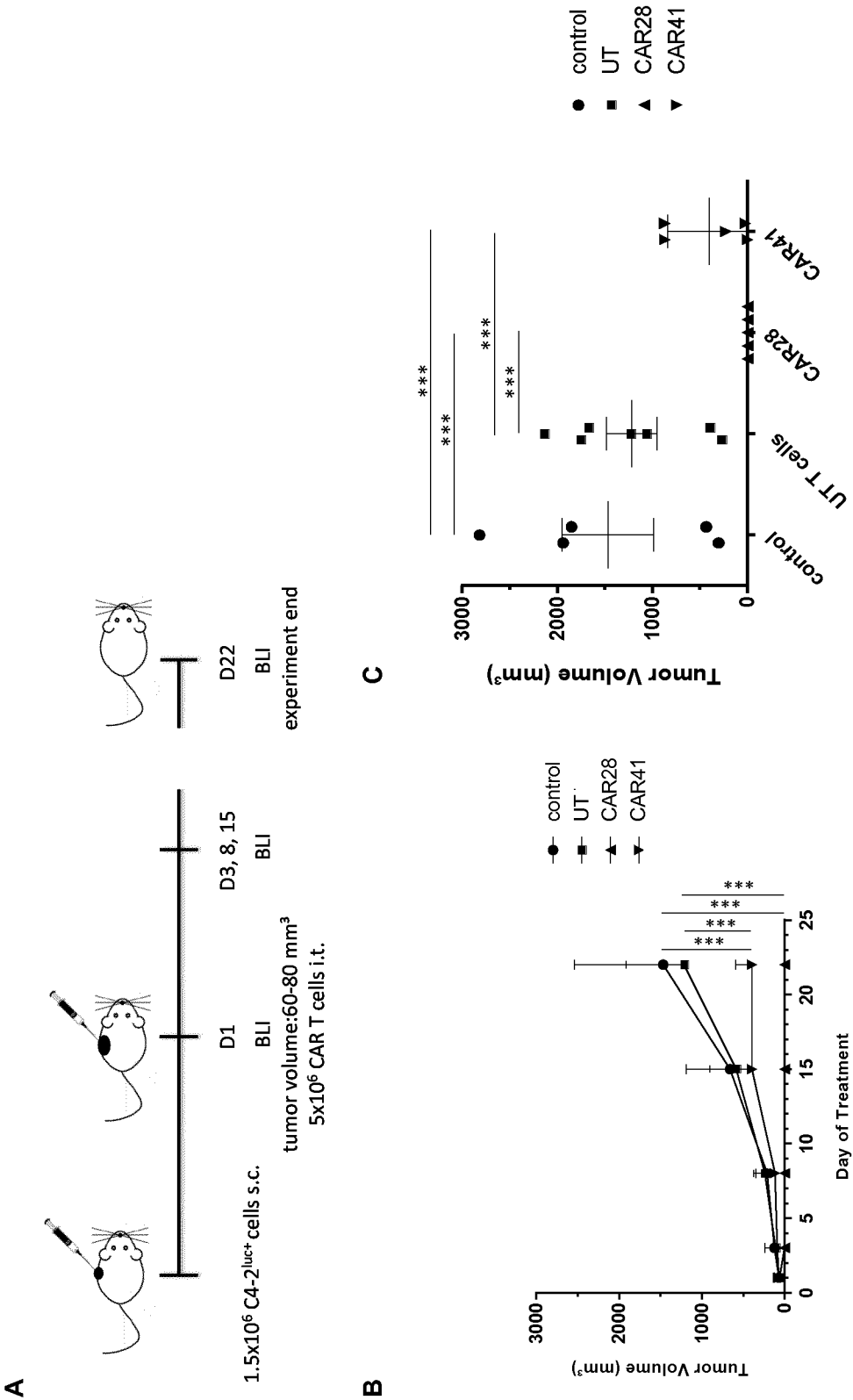


Fig.6



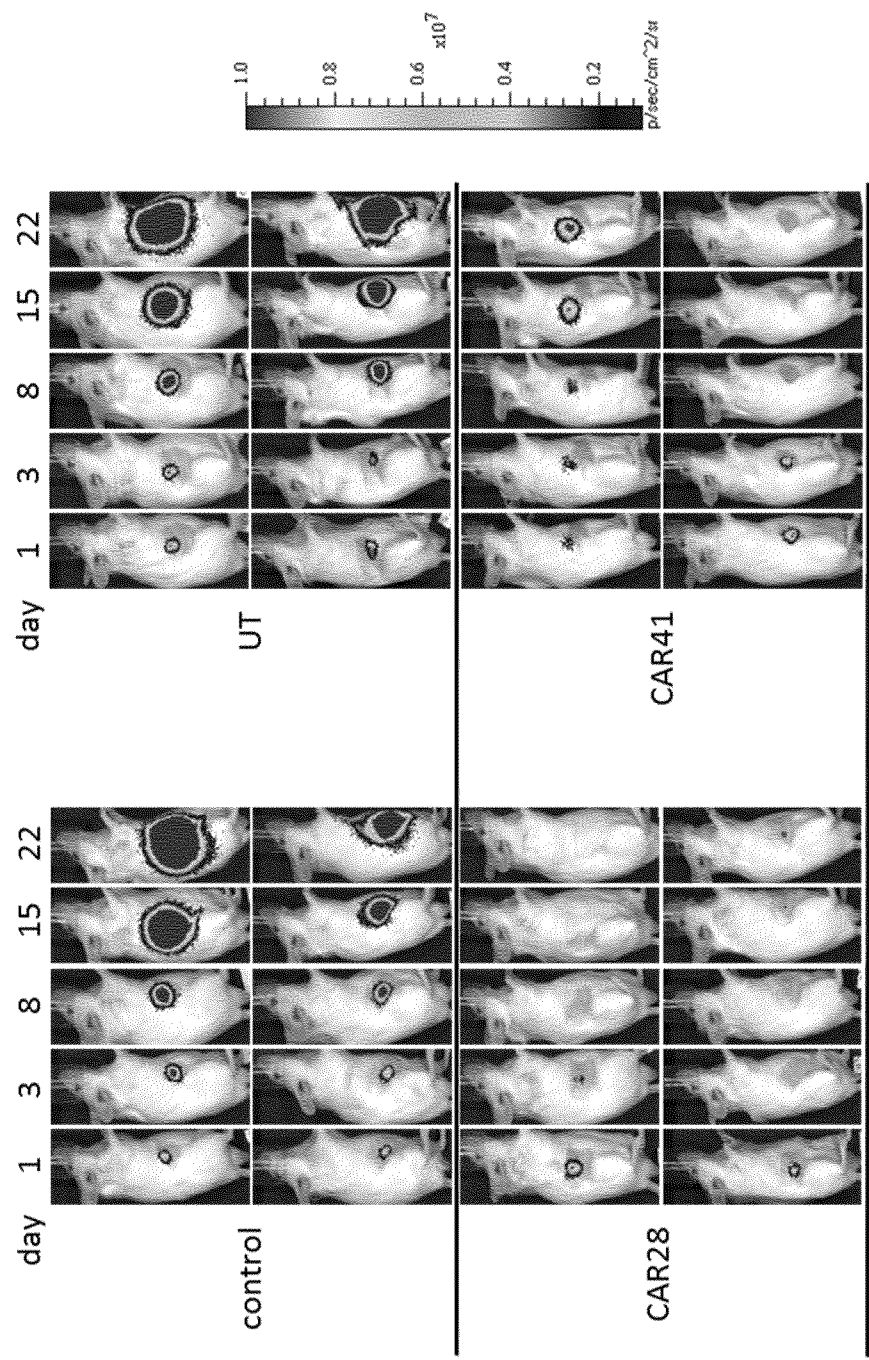


Fig. 6
D

Fig.7

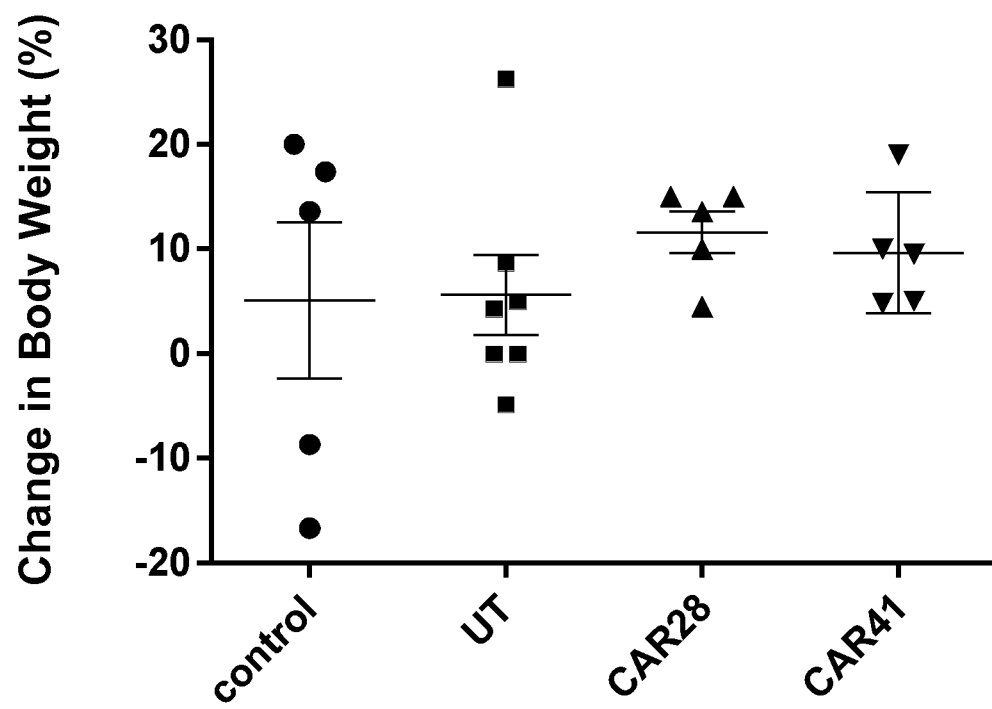
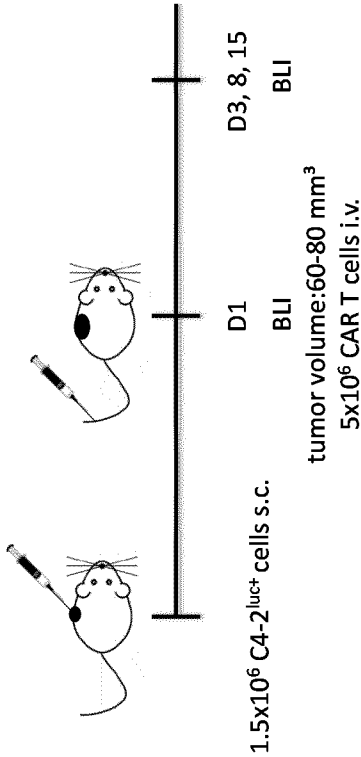
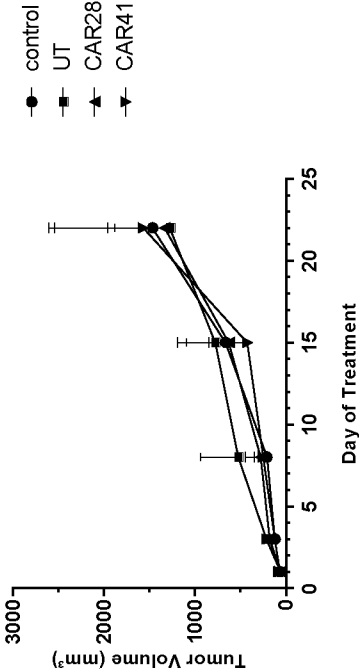


Fig.8

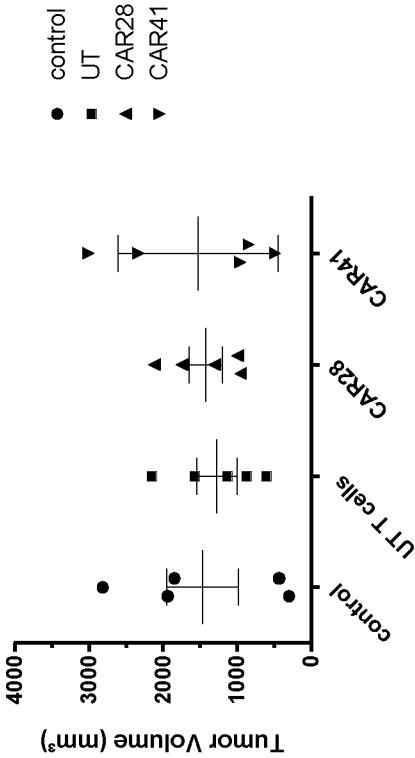
A



B



C



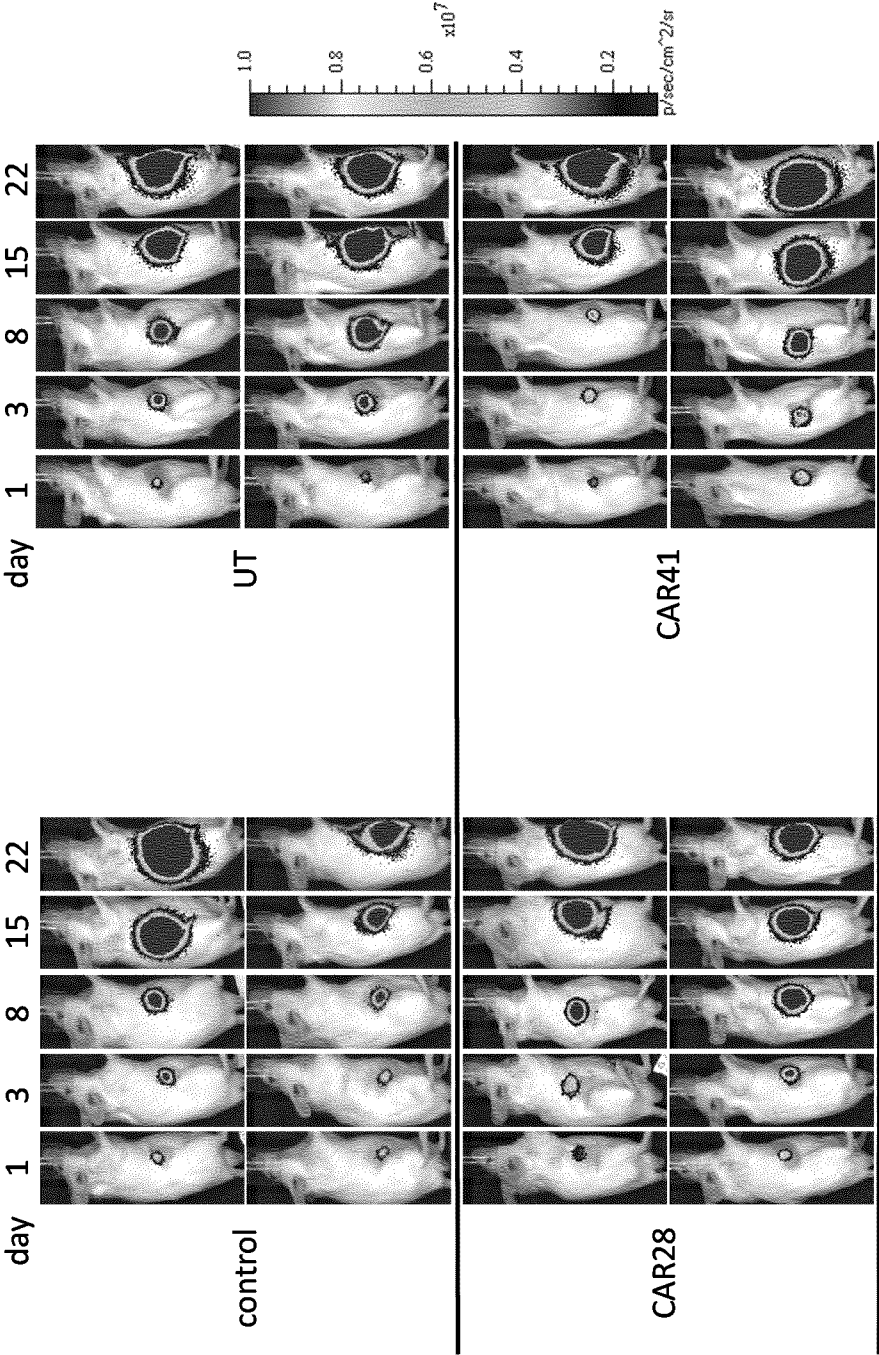
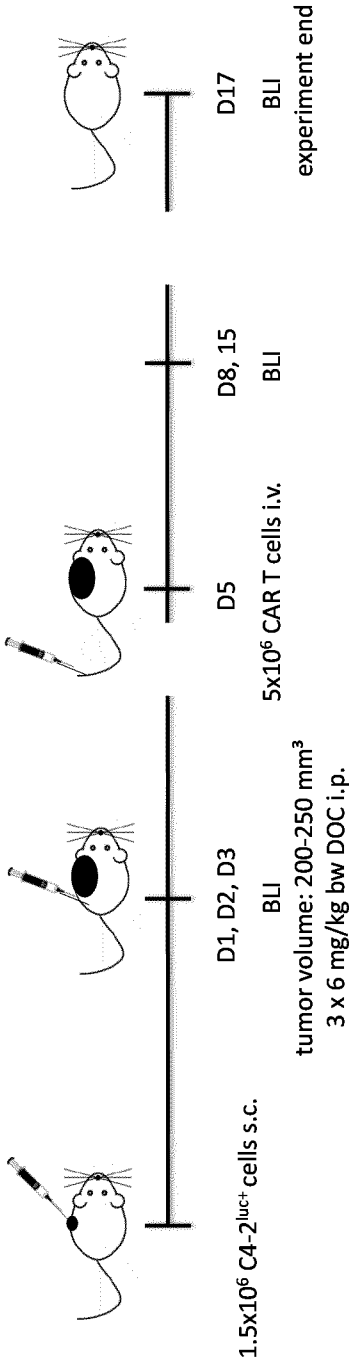


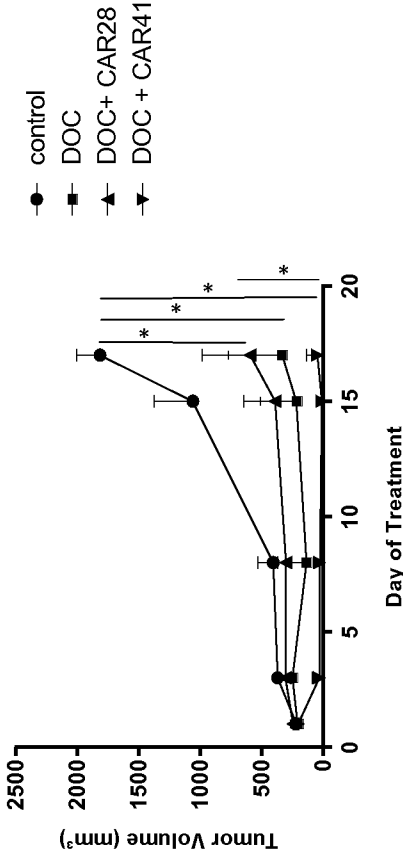
Fig. 8
D

Fig.9

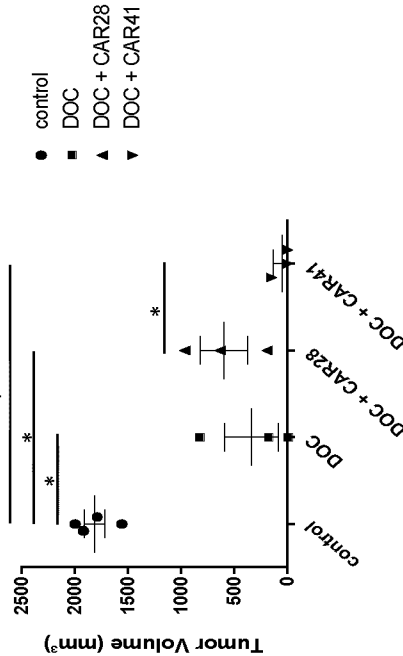
A



B



C



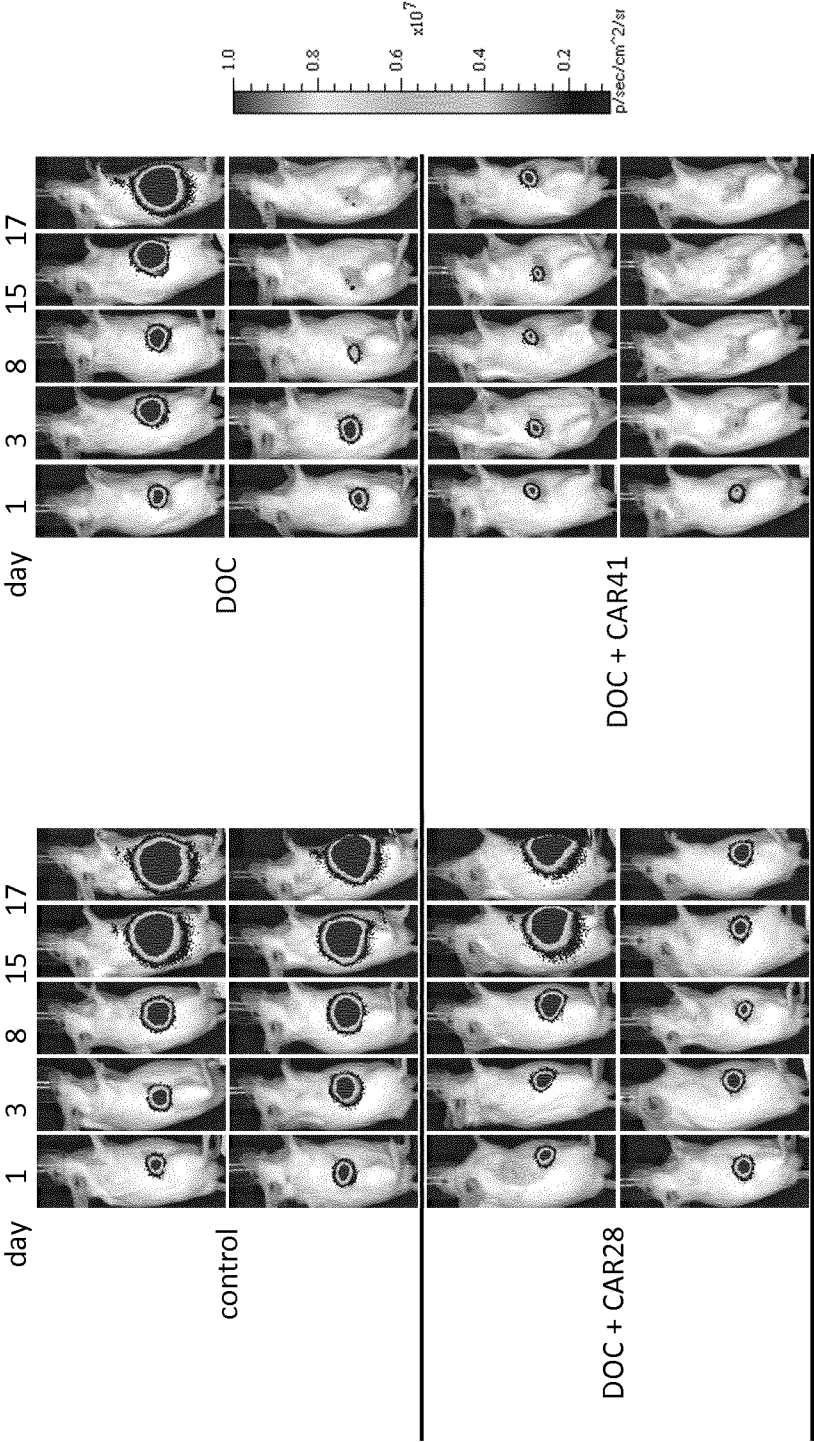


Fig. 9

D

	1	10	20	30	40	50	60	70	80	90	CDR-H1	CDR-H2	CDR-H3	CDR-H4	CDR-H5	CDR-H6	CDR-H7	CDR-H8	CDR-H9	CDR-H10	CDR-H11	CDR-H12	CDR-H13	CDR-H14	CDR-H15	CDR-H16	CDR-H17	CDR-H18	CDR-H19	CDR-H20	CDR-H21	CDR-H22	CDR-H23	CDR-H24	CDR-H25	CDR-H26	CDR-H27	CDR-H28	CDR-H29	CDR-H30	CDR-H31	CDR-H32	CDR-H33	CDR-H34	CDR-H35	CDR-H36	CDR-H37	CDR-H38	CDR-H39	CDR-H40	CDR-H41	CDR-H42	CDR-H43	CDR-H44	CDR-H45	CDR-H46	CDR-H47	CDR-H48	CDR-H49	CDR-H50	CDR-H51	CDR-H52	CDR-H53	CDR-H54	CDR-H55	CDR-H56	CDR-H57	CDR-H58	CDR-H59	CDR-H60	CDR-H61	CDR-H62	CDR-H63	CDR-H64	CDR-H65	CDR-H66	CDR-H67	CDR-H68	CDR-H69	CDR-H70	CDR-H71	CDR-H72	CDR-H73	CDR-H74	CDR-H75	CDR-H76	CDR-H77	CDR-H78	CDR-H79	CDR-H80	CDR-H81	CDR-H82	CDR-H83	CDR-H84	CDR-H85	CDR-H86	CDR-H87	CDR-H88	CDR-H89	CDR-H90	CDR-H91	CDR-H92	CDR-H93	CDR-H94	CDR-H95	CDR-H96	CDR-H97	CDR-H98	CDR-H99	CDR-H100	CDR-H101	CDR-H102	CDR-H103	CDR-H104	CDR-H105	CDR-H106	CDR-H107	CDR-H108	CDR-H109	CDR-H110	CDR-H111	CDR-H112	CDR-H113	CDR-H114	CDR-H115	CDR-H116	CDR-H117	CDR-H118	CDR-H119	CDR-H120	CDR-H121	CDR-H122	CDR-H123	CDR-H124	CDR-H125	CDR-H126	CDR-H127	CDR-H128	CDR-H129	CDR-H130	CDR-H131	CDR-H132	CDR-H133	CDR-H134	CDR-H135	CDR-H136	CDR-H137	CDR-H138	CDR-H139	CDR-H140	CDR-H141	CDR-H142	CDR-H143	CDR-H144	CDR-H145	CDR-H146	CDR-H147	CDR-H148	CDR-H149	CDR-H150	CDR-H151	CDR-H152	CDR-H153	CDR-H154	CDR-H155	CDR-H156	CDR-H157	CDR-H158	CDR-H159	CDR-H160	CDR-H161	CDR-H162	CDR-H163	CDR-H164	CDR-H165	CDR-H166	CDR-H167	CDR-H168	CDR-H169	CDR-H170	CDR-H171	CDR-H172	CDR-H173	CDR-H174	CDR-H175	CDR-H176	CDR-H177	CDR-H178	CDR-H179	CDR-H180	CDR-H181	CDR-H182	CDR-H183	CDR-H184	CDR-H185	CDR-H186	CDR-H187	CDR-H188	CDR-H189	CDR-H190	CDR-H191	CDR-H192	CDR-H193	CDR-H194	CDR-H195	CDR-H196	CDR-H197	CDR-H198	CDR-H199	CDR-H200	CDR-H201	CDR-H202	CDR-H203	CDR-H204	CDR-H205	CDR-H206	CDR-H207	CDR-H208	CDR-H209	CDR-H210	CDR-H211	CDR-H212	CDR-H213	CDR-H214	CDR-H215	CDR-H216	CDR-H217	CDR-H218	CDR-H219	CDR-H220	CDR-H221	CDR-H222	CDR-H223	CDR-H224	CDR-H225	CDR-H226	CDR-H227	CDR-H228	CDR-H229	CDR-H230	CDR-H231	CDR-H232	CDR-H233	CDR-H234	CDR-H235	CDR-H236	CDR-H237	CDR-H238	CDR-H239	CDR-H240	CDR-H241	CDR-H242	CDR-H243	CDR-H244	CDR-H245	CDR-H246	CDR-H247	CDR-H248	CDR-H249	CDR-H250	CDR-H251	CDR-H252	CDR-H253	CDR-H254	CDR-H255	CDR-H256	CDR-H257	CDR-H258	CDR-H259	CDR-H260	CDR-H261	CDR-H262	CDR-H263	CDR-H264	CDR-H265	CDR-H266	CDR-H267	CDR-H268	CDR-H269	CDR-H270	CDR-H271	CDR-H272	CDR-H273	CDR-H274	CDR-H275	CDR-H276	CDR-H277	CDR-H278	CDR-H279	CDR-H280	CDR-H281	CDR-H282	CDR-H283	CDR-H284	CDR-H285	CDR-H286	CDR-H287	CDR-H288	CDR-H289	CDR-H290	CDR-H291	CDR-H292	CDR-H293	CDR-H294	CDR-H295	CDR-H296	CDR-H297	CDR-H298	CDR-H299	CDR-H300	CDR-H301	CDR-H302	CDR-H303	CDR-H304	CDR-H305	CDR-H306	CDR-H307	CDR-H308	CDR-H309	CDR-H310	CDR-H311	CDR-H312	CDR-H313	CDR-H314	CDR-H315	CDR-H316	CDR-H317	CDR-H318	CDR-H319	CDR-H320	CDR-H321	CDR-H322	CDR-H323	CDR-H324	CDR-H325	CDR-H326	CDR-H327	CDR-H328	CDR-H329	CDR-H330	CDR-H331	CDR-H332	CDR-H333	CDR-H334	CDR-H335	CDR-H336	CDR-H337	CDR-H338	CDR-H339	CDR-H340	CDR-H341	CDR-H342	CDR-H343	CDR-H344	CDR-H345	CDR-H346	CDR-H347	CDR-H348	CDR-H349	CDR-H350	CDR-H351	CDR-H352	CDR-H353	CDR-H354	CDR-H355	CDR-H356	CDR-H357	CDR-H358	CDR-H359	CDR-H360	CDR-H361	CDR-H362	CDR-H363	CDR-H364	CDR-H365	CDR-H366	CDR-H367	CDR-H368	CDR-H369	CDR-H370	CDR-H371	CDR-H372	CDR-H3
--	---	----	----	----	----	----	----	----	----	----	--------	--------	--------	--------	--------	--------	--------	--------	--------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	---------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	----------	--------

Fig. 11

	CDR-H1					CDR-H2										CDR-H3																							
	murine FR-1	Y	F	D	I	N	murine FR-2	G	I	S	P	G	D	G	N	T	N	Y	N	E	N	F	K	G	murine FR-3	D	G	N	F	P	Y	A	M	D	S				
D7 VH murine																																							murine FR-4
D7 VH hum 1	human FR-1	Y	F	D	I	N	human FR-2	G	I	S	P	G	D	G	N	T	N	Y	N	E	N	F	K	G	human FR-3	D	G	N	F	P	Y	Y	A	M	D	S	human FR-4		
D7 VH hum 2	human FR-1	Y	F	D	I	N	human FR-2	G	I	S	P	G	D	G	N	T	N	Y	X	X	X	F	X	G	human FR-3	D	G	N	F	P	Y	Y	A	M	D	S	human FR-4		
D7 VH hum 3	human FR-1	Y	F	D	I	N	human FR-2	G	I	S	P	G	D	G	N	X	N	Y	X	X	X	F	X	G	human FR-3	D	G	N	F	P	Y	Y	A	M	D	S	human FR-4		
D7 VH hum 4	human FR-1	Y	F	D	I	N	human FR-2	G	I	S	P	G	D	X	N	X	N	Y	X	X	X	F	X	G	human FR-3	D	G	N	F	P	Y	Y	A	M	D	S	human FR-4		

amino acid replacement

F: framework sequence

		CDR-L1										CDR-L2					CDR-L3																	
		R	S	S	Q	S	L	V	H	S	N	G	N	T	Y	L	H	murine FR-2	T	V	S	N	R	F	S	murine FR-3	S	Q	S	T	H	V	P	T
D7 VL murine	murine FR-1																	murine FR-2								murine FR-3								
D7 VL hum 1	human FR-1	R	S	S	Q	S	L	V	H	S	N	G	N	T	Y	L	H	human FR-2	T	V	S	N	R	F	S	human FR-3	S	Q	S	T	H	V	P	T
D7 VL hum 2	human FR-1	R	S	S	Q	S	L	V	H	S	N	G	N	X	T	Y	L	human FR-2	T	V	S	N	R	F	S	human FR-3	S	Q	S	T	H	V	P	T
D7 VL hum 3	human FR-1	R	S	S	Q	S	L	V	H	S	X	G	X	T	Y	L	H	human FR-2	T	V	S	N	R	F	S	human FR-3	S	Q	S	T	H	V	P	T
D7 VL hum 4	human FR-1	R	S	S	Q	S	L	V	H	S	X	G	X	T	Y	L	H	human FR-2	T	V	S	N	R	X	S	human FR-3	S	Q	S	T	H	V	P	T

amino acid replacement

X1, X4, X6, X7, X8: hydrophilic, uncharged amino acids [serine (S), threonine (T), asparagine (N), glutamine (Q)]
 X2, X3, X9: aliphatic, uncharged amino acids [glycine (G), alanine (A), valine (V), leucine (L), isoleucine (I), proline (P)]
 X5: basic amino acids [(histidine (H), lysine (K), arginine (R))]

Fig. 12a

```

                                IgKappa Leader
                                ~~~~~
      M D F Q V Q I F S F L L I S A S V I M S
1  ATGGATTTTC AGGTGCAGAT TTTCAGCTTC CTGCTAATCA GTGCCTCAGT CATAATGTCT
   TACCTAAAAG TCCACGTCTA AAAGTCGAAG GACGATTAGT CACGGAGTCA GTATTACAGA
                                scFv D7 codon splice optimized
                                ~~~~~
                                VH-D7
                                ~~~~~
IgKappa Leader
~~~
      R M A Q V Q L Q Q S G A E L V E P G A S
61 AGAATGGCCC AGGTGCAGCT TCAACAGTCT GCGCGGAGC TGGTCGAGCC CGGTGCTAGC
   TCTTACCGGG TCCACGTCTA AGTTGTCAGA CCGCGCCTCG ACCAGCTCGG GCCACGATCG
                                VH-D7
                                ~~~~~
                                CDR-H1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      V K L S C K A S G Y T F T Y F D I N W L
121 GTTAAGCTGA GTTGAAGGC GTCCGGCTAC ACATTACCT ACTTCGATAT CAATTGGCTG
   CAATTGCACT CAACATTCGG CAGGCCGATG TGTAAATGGA TGAAGCTATA GTTAACCGAC
                                VH-D7
                                ~~~~~
                                CDR-H2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      R Q R P E Q G L E W I G G I S P G D G N
181 AGACAAAGGC CTGAACAGGG CCTGGAATGG ATCGGCGGAA TCTCTCCCGG AGATGGGAAT
   TCTGTTTCCG GACTTGTCCT GGACCTTACC TAGCCGCTTT AGAGAGGGCC TCTACCTTAA
                                VH-D7
                                ~~~~~
                                CDR-H2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      T N Y N E N F K G K A T L T I D K S S T
241 ACAAATTATA ACGAGAACTT CAAGGGAAGG GCTACTCTTA CTATAGACAA GTCCAGCACT
   TGTTTAATAT TGCTCTTGAA GTTCCCTTTC CGATGAGAAT GATATCTGTT CAGGTCGTGA
                                VH-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      T A Y I Q L S R L T S E D S A V Y F C A
301 ACGGCCTACA TCCAGCTGTC CCGCCTCACG AGCGAGGACA GCGCCGTGTA TTTCTGTGCA
   TGCCGGATGT AGGTCGACAG GCGGAGTGC TCGCTCCTGT CGCGGCACAT AAAGACACGT
                                VH-D7
                                ~~~~~
                                CDR-H3
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      R D G N F P Y Y A M D S W G Q G T S V T
361 AGGGACGGCA ACTTCCCCTA CTATGCAATG GATTCTTGGG GGCAGGGCAC TTCCGTCACA
   TCCCTGCCGT TGAAGGGGAT GATACGTTAC CTAAGAACCC CCGTCCCGTG AAGGCAGTGT
                                VH-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      V S S A K T T P K L G G G G S G G G G S
421 GTGAGCTCTG CCAAGACCAC ACCGAACTG GCGGAGGAG GCAGTGGAGG TGGGGGGAGC
   CACTCGAGAC GGTCTGGTG TGGCTTTGAC CCGCCTCCTC CGTCACTTCC ACCCCCTCG
                                VL-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      G G G G S A R V D I E L T Q S P L S L P
481 GGGGGTGGGG GAAGTGCTCG CGTGGATATT GAGCTCACTC AGAGTCCTCT GTCTCTCCCT
   CCCCCACCC CTTCACGAGC GCACCTATAA CTCGAGTGAG TCTCAGGAGA CAGAGAGGGA
                                VL-D7
                                ~~~~~

```

Fig. 12b

```

                                CDR-L1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
541  V I L G D Q A S I S C R S S Q S L V H S
    GTGATACTGG GCGACCAAGC TAGCATTAGC TGCCGAAGCA GCCAATCACT GGTCCACTCT
    CACTATGACC CGCTGGTTCG ATCGTAATCG ACGGCTTCGT CGGTTAGTGA CCAGGTGAGA
                                VL-D7
                                ~~~~~
                                CDR-L1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
601  N G N T Y L H W F L Q K P G Q S P K L L
    AACGGAAACA CCTATCTTCA CTGGTTTCTC CAAAAGCCTG GACAGTCCCC GAAGTTGCTT
    TTGCCTTTGT GGATAGAAAGT GACCAAAGAG GTTTTCGGAC CTGTCAGGGG CTTCAACGAA
                                VL-D7
                                ~~~~~
                                CDR-L2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
661  I Y T V S N R F S G V P D R F S G S G S
    ATTTATACTG TCAGCAACCG ATTCTCAGGG GTCCCCGATC GATTACGCGG CAGCGGGAGC
    TAAATATGAC AGTCGTTGGC TAAGAGTCCC CAGGGGCTAG CTAAGTCGCC GTCGCCCTCG
                                VL-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
721  G T D F T L K I S R V E A E D L G V Y F
    GGGACCGACT TTACCCTCAA GATCTCCCGC GTGGAGGCCG AGGACCTGGG AGTCTATTTT
    CCCTGGCTGA AATGGGAGTT CTAGAGGGCG CACCTCCGGC TCCTGGACCC TCAGATAAAG
                                VL-D7
                                ~~~~~
                                CDR-L3
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
781  C S Q S T H V P T F G G G T K L E I K R
    TGCAGCCAGT CTACTCATGT GCCGACGTTT GGAGGAGGGA CGAAGTTGGA GATAAAAAGA
    ACGTCGGTCA GATGAGTACA CGGCTGCAAG CCTCCTCCCT GCTTCAACCT CTATTTTTCT
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
841  S D P A E P K S P D K T H T C P P C P A
    TCGGATCCCG CCGAGCCCAA ATCTCCTGAC AAAACTCACA CATGCCACCC GTGCCAGCA
    AGCCTAGGGC GGCTCGGGTT TAGAGGACTG TTTTGAGTGT GTACGGGTGG CACGGGTCGT
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
901  P P V A G P S V F L F P P K P K D T L M
    CCTCCAGTCG CGGGACCGTC AGTCTTCCTC TTCCCCCAA AACCAAGGA CACCCTCATG
    GGAGGTCAGC GCCCTGGCAG TCAGAAGGAG AAGGGGGGTT TTGGGTTCCT GTGGGAGTAC
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
961  I A R T P E V T C V V V D V S H E D P E
    ATGCCCCGGA CCCCTGAGGT CACATGCGTG GTGGTGGACG TGAGCCACGA AGACCCTGAG
    TAGCGGGCCT GGGGACTCCA GTGTACGCAC CACCACCTGC ACTCGGTGCT TCTGGGACTC
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1021 V K F N W Y V D G V E V H N A K T K P R
    GTCAAGTTCA ACTGGTACGT GGACGGCGTG GAGGTGCATA ATGCCAAGAC AAAGCCGCGG
    CAGTTCAAGT TGACCATGCA CTGCGGCAC CTCCACGTAT TACGGTTCTG TTTCGGCGCC
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1081 E E Q Y N S T Y R V V S V L T V L H Q D
    GAGGAGCAGT ACAACAGCAC GTACCGTGTG GTCAGCGTCC TCACCGTCCT GCACCAGGAC
    CTCCTCGTCA TGTGTCTGTG CATGGACAC CAGTCGCAGG AGTGGCAGGA CGTGGTCTGT
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1141 W L N G K E Y K C K V S N K A L P A P I
    TGCTGAATG GCAAGGAGTA CAAGTGCAAG GTCTCCAACA AAGCCCTCCC AGCCCCATC
    ACCGACTTAC CGTTCTCAT GTTACGTTT CAGAGTTGT TTCGGGAGGG TCGGGGGTAG
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~

```

Fig. 12c

```

E K T I S K A K G Q P R E P Q V Y T L P
1201 GAGAAAACCA TCTCCAAAGC CAAAGGGCAG CCCCAGAAC CACAGGTGTA CACCCGTGCC
CTCTTTTGGT AGAGGTTCG GTTCCCGTC GGGGCTCTTG GTGTCCACAT GTGGGACGGG
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
P S R D E L T K N Q V S L T C L V K G F
1261 CCATCCCGGG ATGAGCTGAC CAAGAACCAG GTCAGCCTGA CCTGCCTGGT CAAAGGCTTC
GGTAGGGCCC TACTCGACTG GTTCTTGCTC CAGTCGGACT GGACGGACCA GTTCCGAAG
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
Y P S D I A V E W E S N G Q P E N N Y K
1321 TATCCCAGCG ACATCGCCGT GGAGTGGGAG AGCAATGGGC AGCCGGAGAA CAACTACAAG
ATAGGGTCGC TGTAGCGGCA CCTCACCTC TCGTTACCCG TCGGCCTCTT GTTGATGTTT
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
T T P P V L D S D G S F F L Y S K L T V
1381 ACCACGCCCTC CCGTGCTGGA CTCCGACGGC TCCTTCTTCC TCTACAGCAA GCTCACCGTG
TGGTGGCGAG GGCACGACCT GAGGCTGCCG AGGAAGAAGG AGATGTCGTT CGAGTGGCAC
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
D K S R W Q Q G N V F S C S V M H E A L
1441 GACAAGAGCA GGTGGCAGCA GGGGAACGTC TTCTCATGCT CCGTGATGCA TGAGGCTCTG
CTGTTCTCGT CCACCGTCGT CCCCTTGCA GAGAGTACGA GGCACACTACGT ACTCCGAGAC
human CD28 deltaLck TM+IC
~~~~~
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
H N H Y T Q K S L S L S P G K K D P K F
1501 CACAACCACT ACACGCAGAA GAGCCTCTCC CTGTCTCCGG GTAAAAAAGA TCCCAAATTT
GTGTTGGTGA TGTGCGTCTT CTCGGAGAGG GACAGAGGCC CATTTTTTCT AGGGTTTAA
human CD28 deltaLck TM+IC
~~~~~
W V L V V V G G V L A C Y S L L V T V A
1561 TGGGTGCTGG TGGTGGTTGG TGGAGTCCTG GCTTGCTATA GCTTGCTAGT AACAGTGGCC
ACCCACGACC ACCACCAACC ACCTCAGGAC CGAACGATAT CGAACGATCA TTGTCACCGG
human CD28 deltaLck TM+IC
~~~~~
F I I F W V R S K R S R L L H S D Y M N
1621 TTTATTATTT TCTGGGTGAG GAGTAAGAGG AGCAGGCTCC TGCACAGTGA CTACATGAAC
AAATAATAAA AGACCCACTC CTCATTCTCC TCGTCCGAGG ACGTGTCAC TATGTACTTG
human CD28 deltaLck TM+IC
~~~~~
M T P R R P G P T R K H Y Q A Y A A A R
1681 ATGACTCCCC GCCGCCCCGG GCCCACCCGC AAGCATTACC AGGCCTATGC CGCCGCACGC
TACTGAGGGG CGGCGGGGCC CGGGTGGGCG TTCGTAATGG TCCGATACG GCGGCGTGCG
Human CD3zeta
~~~~~
human CD28 deltaLck TM+IC
~~~~~
D F A A Y R S L R V K F S R S A D A P A
1741 GACTTTCGAG CCTATCGCTC CCTGAGAGTG AAGTTCAGCA GGAGCGCAGA CGCCCCCGCG
CTGAAGCGTC GGATAGCGAG GGA CTCTCAC TTCAAGTCGT CCTCGCGTCT GCGGGGGCGC
Human CD3zeta
~~~~~
Y Q Q G Q N Q L Y N E L N L G R R E E Y
1801 TACCAGCAGG GCCAGAACCA GCTCTATAAC GAGCTCAATC TAGGACGAAG AGAGGAGTAC
ATGCTCGTCC CGGTCTTGGT CGAGATATTG CTCGAGTTAG ATCCTGCTTC TCTCCTCATG
Human CD3zeta
~~~~~
D V L D K R R G R D P E M G G K P R R K
1861 GATGTTTTGG ACAAGAGACG TGGCCGGGAC CCTGAGATGG GGGGAAAGCC GAGAAGGAAG
CTACAAAACC TGTTCTCTGC ACCGGCCCTG GGA CTCTACC CCCCTTTCGG CTCTTCCTTC
Human CD3zeta
~~~~~
N P Q E G L Y N E L Q K D K M A E A Y S
1921 AACCCCTCAGG AAGGCCTGTA CAATGAAGT CAGAAAGATA AGATGGCGGA GGCCTACAGT
TTGGGAGTCC TTCCGGACAT GTTACTTGAC GTCTTTCTAT TCTACCGCCT CCGGATGTCA
Human CD3zeta
~~~~~
E I G M K G E R R R G K G H D G L Y Q G
1981 GAGATTGGGA TGAAGGCGA GCGCCGGAGG GGCAAGGGC ACGATGGCCT TTACAGGGT
CTCTAACCTT ACTTTCCGCT CGCGGCTCC CCGTTCCCCG TGCTACCGGA AATGGTCCCA
Human CD3zeta

```

Fig. 12d

```
~~~~~  
      L S T A T K D T Y D A L H M Q A L P P R  
2041 CTCAGTACAG CCACCAAGGA CACCTACGAC GCCCTTCACA TGCAGGCCCT GCCCCCTCGC  
      GAGTCATGTC GGTGGTTCCT GTGGATGCTG CGGGAAGTGT ACGTCCGGGA CGGGGGAGCG  
      Human CD3zeta  
      ~~~  
      *  
2101 TAA  
      ATT
```

Fig. 13a

```

                                IgKappa Leader
                                ~~~~~
      M D F Q V Q I F S F L L I S A S V I M S
1  ATGGATTTTC AGGTGCAGAT TTTCAGCTTC CTGCTAATCA GTGCCTCAGT CATAATGTCT
   TACCTAAAAG TCCACGTCTA AAAGTCGAAG GACGATTAGT CACGGAGTCA GTATTACAGA
                                scFv D7 codon splice optimized
                                ~~~~~
                                VH-D7
                                ~~~~~
IgKappa Leader
~~~
      R M A Q V Q L Q Q S G A E L V E P G A S
61 AGAATGGCCC AGGTGCAGCT TCAACAGTCT GGC GCGGAGC TGGTCGAGCC CGGTGCTAGC
   TCTTACCGGG TCCACGTCTA AGTTGTCAGA CCGCGCCTCG ACCAGCTCGG GCCACGATCG
                                VH-D7
                                ~~~~~
                                CDR-H1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      V K L S C K A S G Y T F T Y F D I N W L
121 GTTAAGCTGA GTTGTAAAGC GTCCGGCTAC ACATTACCT ACTTCGATAT CAATTGGCTG
   CAATTGCACT CAACATTCGG CAGGCCGATG TGTAATGGA TGAAGCTATA GTTAACCGAC
                                VH-D7
                                ~~~~~
                                CDR-H2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      R Q R P E Q G L E W I G G I S P G D G N
181 AGACAAAGGC CTGAACAGGG CCTGGAATGG ATCGGCGGAA TCTCTCCCGG AGATGGGAAT
   TCTGTTTCCG GACTTGTCCT GGACCTTACC TAGCCGCTTT AGAGAGGGCC TCTACCTTAA
                                VH-D7
                                ~~~~~
                                CDR-H2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      T N Y N E N F K G K A T L T I D K S S T
241 ACAAATTATA ACGAGAACTT CAAGGGAAGG GCTACTCTTA CTATAGACAA GTCCAGCACT
   TGTTTAATAT TGCTCTTGAA GTTCCCTTTC CGATGAGAAT GATATCTGTT CAGGTCGTGA
                                VH-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      T A Y I Q L S R L T S E D S A V Y F C A
301 ACGGCCTACA TCCAGCTGTC CCGCCTCACG AGCGAGGACA GCGCCGTGTA TTTCTGTGCA
   TGCCGGATGT AGGTCGACAG GCGGAGTGC TCGCTCCTGT CGCGGCACAT AAAGACACGT
                                VH-D7
                                ~~~~~
                                CDR-H3
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      R D G N F P Y Y A M D S W G Q G T S V T
361 AGGGACGGCA ACTTCCCCTA CTATGCAATG GATTCTGGG GGCAGGGCAC TTCCGTCACA
   TCCCTGCCGT TGAAGGGGAT GATACGTTAC CTAAGAACCC CCGTCCCGTG AAGGCAGTGT
                                VH-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      V S S A K T T P K L G G G G S G G G G S
421 GTGAGCTCTG CCAAGACCAC ACCGAACTG GCGGAGGAG GCAGTGGAGG TGGGGGGAGC
   CACTCGAGAC GGTCTGGTG TGGCTTTGAC CCGCCTCCTC CGTCACCTCC ACCCCCTCG
                                VL-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
      G G G G S A R V D I E L T Q S P L S L P
481 GGGGGTGGGG GAAGTGCTCG CGTGGATATT GAGCTCACTC AGAGTCCTCT GTCTCTCCCT
   CCCCCACCC CTTCACGAGC GCACCTATAA CTCGAGTGAG TCTCAGGAGA CAGAGAGGGA
                                VL-D7
                                ~~~~~

```

Fig. 13b

```

                                CDR-L1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
541  V I L G D Q A S I S C R S S Q S L V H S
    GTGATACTGG GCGACCAAGC TAGCATTAGC TGCCGAAGCA GCCAATCACT GGTCCACTCT
    CACTATGACC CGCTGGTTCG ATCGTAATCG ACGGCTTCGT CGGTTAGTGA CCAGGTGAGA
                                VL-D7
                                ~~~~~
                                CDR-L1
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
601  N G N T Y L H W F L Q K P G Q S P K L L
    AACGGAAACA CCTATCTTCA CTGGTTTCTC CAAAAGCCTG GACAGTCCCC GAAGTTGCTT
    TTGCCTTTGT GGATAGAAAGT GACCAAAGAG GTTTTCGGAC CTGTCAGGGG CTTCAACGAA
                                VL-D7
                                ~~~~~
                                CDR-L2
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
661  I Y T V S N R F S G V P D R F S G S G S
    ATTTATACTG TCAGCAACCG ATTCTCAGGG GTCCCCGATC GATTACGCGG CAGCGGGAGC
    TAAATATGAC AGTCGTTGGC TAAGAGTCCC CAGGGGCTAG CTAAGTCGCC GTCGCCCTCG
                                VL-D7
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
721  G T D F T L K I S R V E A E D L G V Y F
    GGGACCGACT TTACCCTCAA GATCTCCCGC GTGGAGGCCG AGGACCTGGG AGTCTATTTT
    CCCTGGCTGA AATGGGAGTT CTAGAGGGCG CACCTCCGGC TCCTGGACCC TCAGATAAAG
                                VL-D7
                                ~~~~~
                                CDR-L3
                                ~~~~~
                                scFv D7 codon splice optimized
                                ~~~~~
781  C S Q S T H V P T F G G G T K L E I K R
    TGCAGCCAGT CTACTCATGT GCCGACGTTT GGAGGAGGGA CGAAGTTGGA GATAAAAAGA
    ACGTCGTGCA GATGAGTACA CGGCTGCAAG CCTCCTCCCT GCTTCAACCT CTATTTTTCT
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
841  A A A L P A E P K S P D K T H T C P P C
    GCGGCCGCTC TACCCGCCGA GCCCAAATCT CCTGACAAAA CTCACACATG CCCACCGTGC
    CGCCGGCGAG ATGGGCGGCT CGGGTTTAGA GGAAGTGTTC GAGTGTGTAC GGGTGGCAGC
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
901  P A P P V A G P S V F L F P P K P K D T
    CCAGCACCTC CAGTCGCGGG ACCGTCAGTC TTCCTCTTCC CCCCCAAACC CAAGGACACC
    GGTCGTGAG GTCAGCGCCC TGCCAGTCAG AAGGAGAAGG GGGGTTTGG GTTCCTGTGG
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
961  L M I A R T P E V T C V V V D V S H E D
    LTCATGATCG CCCGGACCCC TGAGGTCACA TGCGTGGTGG TGGACGTGAG CCACGAAGAC
    GAGTACTAGC GGGCCTGGGG ACTCCAGTGT ACGCACCACC ACCTGCACTC GGTGCTTCTG
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1021 P E V K F N W Y V D G V E V H N A K T K
    CCTGAGGTCA AGTTCAACTG GTACGTGGAC GGCCTGGAGG TGCATAATGC CAAGACAAAG
    GGACTCCAGT TCAAGTTGAC CATGCACCTG CCGCACCTCC ACGTATTACG GTTCTGTTTC
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1081 P R E E Q Y N S T Y R V V S V L T V L H
    CCGCGGGAGG AGCAGTACAA CAGCACGTAC CGTGTGGTCA GCGTCCTCAC CGTCCTGCAC
    GCGGCCCTCC TCGTCATGTT GTCGTGCATG GCACACCAGT CGCAGGAGTG GCAGGACGTG
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~
1141 Q D W L N G K E Y K C K V S N K A L P A
    CAGGACTGGC TGAATGGCAA GGAGTACAAG TGCAAGGTCT CCAACAAAGC CCTCCCAGCC
    GTCCTGACCG ACTTACCGTT CCTCATGTTT ACGTTCCAGA GGTGTTTTCG GGAGGGTCGG
                                Human IgG1 Fc Hinge-CH2/CH3 delta
                                ~~~~~

```

Fig. 13c

```

P I E K T I S K A K G Q P R E P Q V Y T
1201 CCCATCGAGA AAACCATCTC CAAAGCCAAA GGGCAGCCCC GAGAACCACA GGTGTACACC
GGGTAGCTCT TTTGGTAGAG GTTTCGGTTT CCCGTCGGGG CTCTTGGTGT CCACATGTGG
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
L P P S R D E L T K N Q V S L T C L V K
1261 CTGCCCCCAT CCCGGGATGA GCTGACCAAG AACCAGGTCA GCCTGACCTG CCTGGTCAAA
GACGGGGGTA GGGCCCTACT CGACTGGTTC TTGGTCCAGT CGGACTGGAC GGACCAGTTT
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
G F Y P S D I A V E W E S N G Q P E N N
1321 GGCTTCTATC CCAGCGACAT CGCCGTGGAG TGGGAGAGCA ATGGGCAGCC GGAGAACAAC
CCGAAGATAG GGTGCTGTGA GCGGCACCTC ACCCTCTCGT TACCCGTCGG CCTCTTGTTC
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
Y K T T P P V L D S D G S F F L Y S K L
1381 TACAAGACCA CGCCTCCCGT GCTGGAATCC GACGGCTCCT TCTTCCTCTA CAGCAAGCTC
ATGTTCTGGT GCGGAGGGCA CGACCTGAGG CTGCCGAGGA AGAAGGAGAT GTCGTTTCGAG
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
T V D K S R W Q Q G N V F S C S V M H E
1441 ACCGTGGACA AGAGCAGGTG GCAGCAGGGG AACGTCTTCT CATGCTCCGT GATGCATGAG
TGGCACCTGT TCTCGTCCAC CGTCGTCCCC TTGCAGAAAG GTACGAGGCA CTACGTACTC
Ser-Insert Human CD8a(tm)
~~~~~
Human IgG1 Fc Hinge-CH2/CH3 delta
~~~~~
A L H N H Y T Q K S L S S L S P G K K I
1501 GCTCTGCACA ACCACTACAC GCAGAAGAGC CTCTCGAGCC TGTCTCCGGG TAAAAAATC
CGAGACGTGT TGGTGATGTG CGTCTTCTCG GAGAGCTCGG ACAGAGGCCC ATTTTCTTTC
Human CD8a(tm)
~~~~~
Y I W A P L A G T C G V L L L S L V I T
1561 TACATCTGGG CGCCCTTGGC CGGGACTTGT GGGGTCCTTC TCCTGTCACT GGTATACACC
ATGTAGACCC GCGGGAACCG GCCCTGAACA CCCAGGAAG AGGACAGTGA CCAATAGTGG
Human CD8a(tm)
~~~~~
Human 4-1BB(ic)
~~~~~
L Y C K R G R K K L L Y I F K Q P F M R
1621 CTTTACTGCA AACGGGGCAG AAAGAAACTC CTGTATATAT TCAAACAACC ATTTATGAGA
GAAATGACGT TTGCCCCGTC TTTCTTTGAG GACATATATA AGTTTGTGTG TAAATACTCT
Human 4-1BB(ic)
~~~~~
P V Q T T Q E E D G C S C R F P E E E E
1681 CCAGTACAAA CTACTCAAGA GGAAGATGGC TGTAGCTGCC GATTTCAGGA AGAAGAAGAA
GGTCATGTTT GATGAGTTCT CCTTCTACCG ACATCGACGG CTAAAGGTCT TCTTCTTCTT
Human CD3zeta
~~~~~
Human 4-1BB(ic)
~~~~~
G G C E L L R V K F S R S A D A P A Y Q
1741 GGAGGATGTG AACTGCTGAG AGTGAAGTTC AGCAGGAGCG CAGACGCCCC CGCGTACCAG
CCTCCTACAC TTGACGACTC TCACTTCAAG TCGTCCTCGC GTCTGCGGGG GCGCATGGTC
Human CD3zeta
~~~~~
Q G Q N Q L Y N E L N L G R R E E Y D V
1801 CAGGGCCAGA ACCAGCTCTA TAACGAGCTC AATCTAGGAC GAAGAGAGGA GTACGATGTT
GTCCCGGTCT TGGTCGAGAT ATTGCTCGAG TTAGATCCTG CTTCTCTCCT CATGCTACAA
Human CD3zeta
~~~~~
L D K R R G R D P E M G G K P R R K N P
1861 TTGGACAAGA GACGTGGCCG GGACCTGAG ATGGGGGAA AGCCGAGAAG GAAGAACCCT
AACCTGTTCT CTGCACCGGC CCTGGGACTC TACCCCTTTC TCGGCTCTTC CTTCTTGGGA
Human CD3zeta
~~~~~
Q E G L Y N E L Q K D K M A E A Y S E I
1921 CAGGAAGGCC TGTACAATGA ACTGCAGAAA GATAAGATGG CGGAGGCCTA CAGTGAGATT
GTCCTTCGGG ACATGTTACT TGACGTCTTT CTATTCTACC GCCTCCGGAT GTCACTCTAA
Human CD3zeta
~~~~~
G M K G E R R R G K G H D G L Y Q G L S
1981 GGGATGAAAG GCGAGCGCCG GAGGGGCAAG GGGCAGATG GCCTTTACCA GGTCTCAGT

```

Fig. 13d

```
CCCTACTTTC CGCTCGCGGC CTCCCCGTTT CCCGTGCTAC CGGAAATGGT CCCAGAGTCA
                                                    STOP
                                                    ~~~
                                Human CD3zeta
~~~~~
  T A T K D T Y D A L H M Q A L P P R *
2041 ACAGCCACCA AGGACACCTA CGACGCCCTT CACATGCAGG CCCTGCCCCC TCGCTAA
    TGTGGTGGT TCCTGTGGAT GTCGCGGAA GTGTACGTCC GGGACGGGGG AGCGATT
```

Fig. 14a

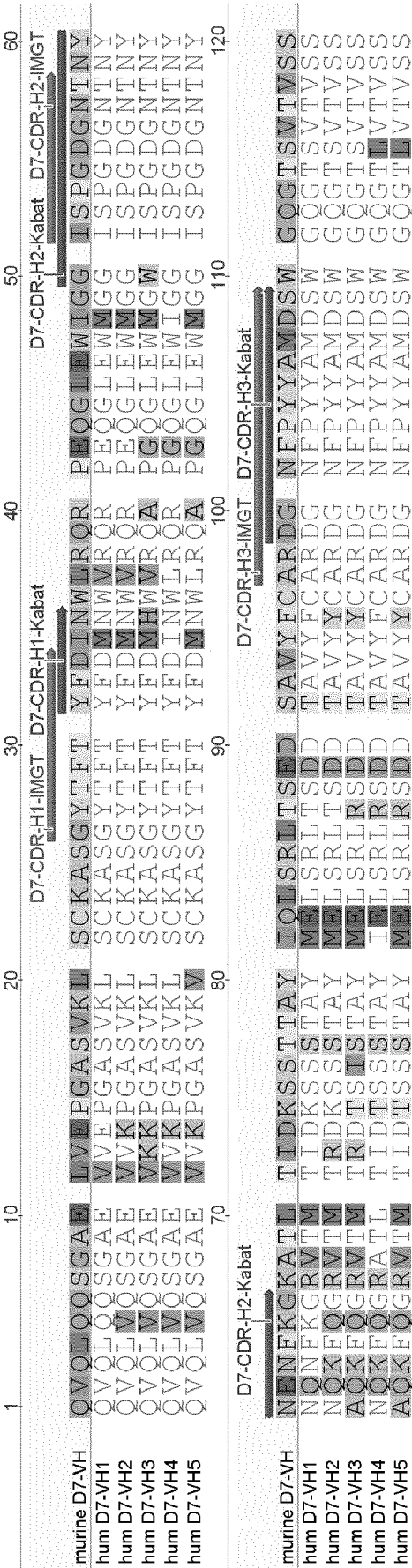


Fig. 14b

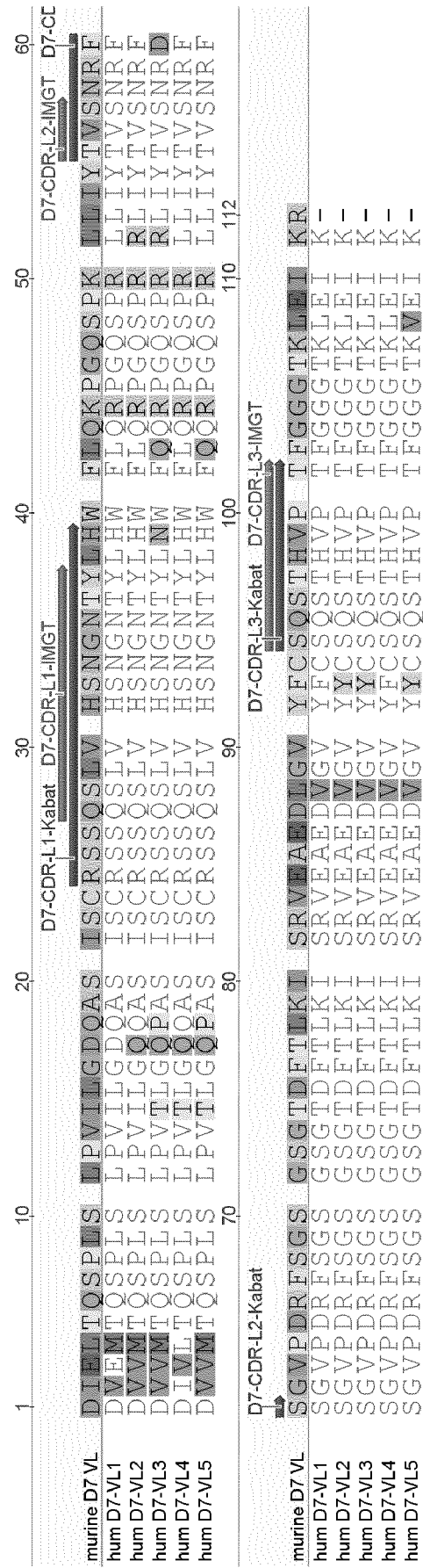


Fig.15

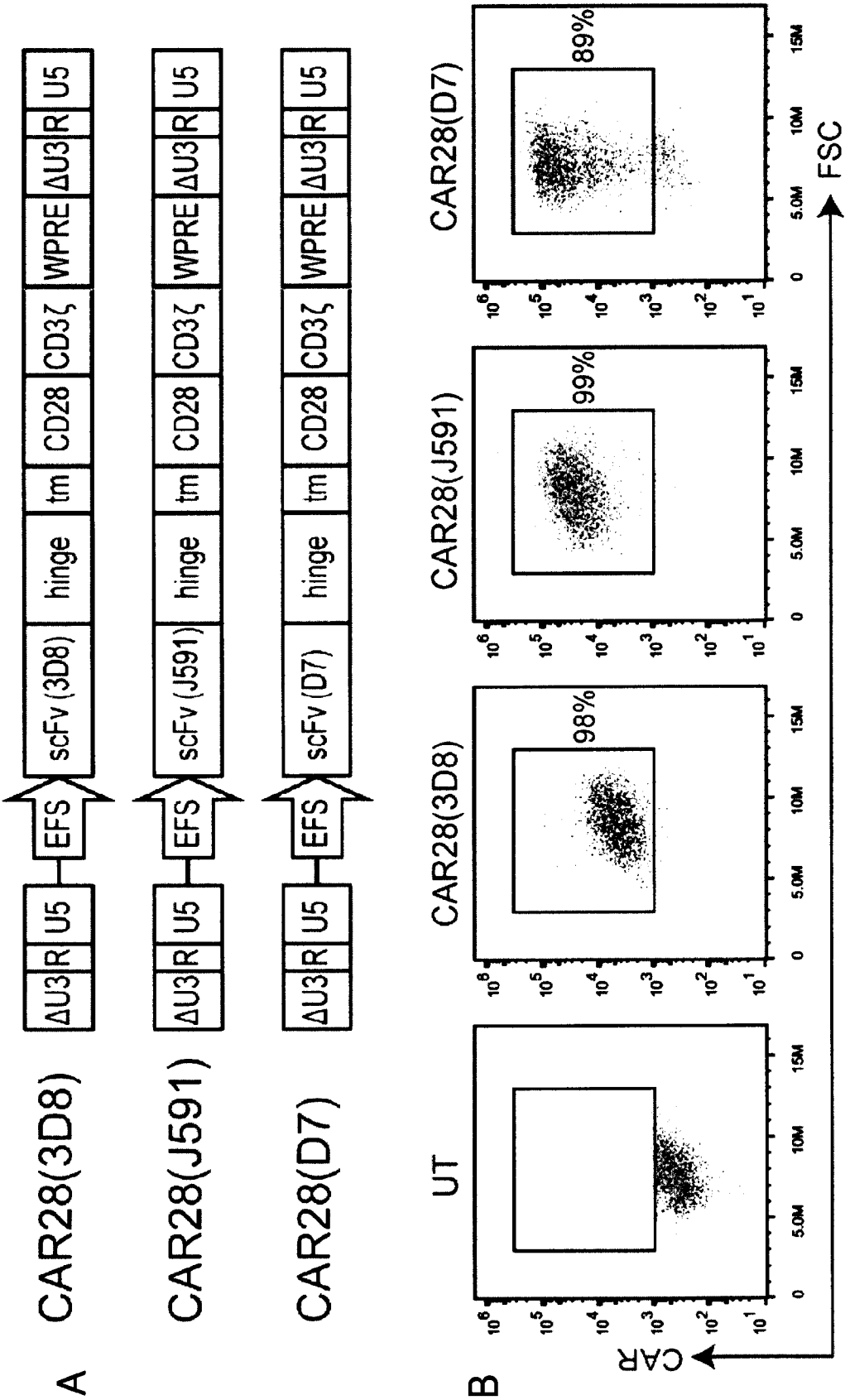


Fig.15

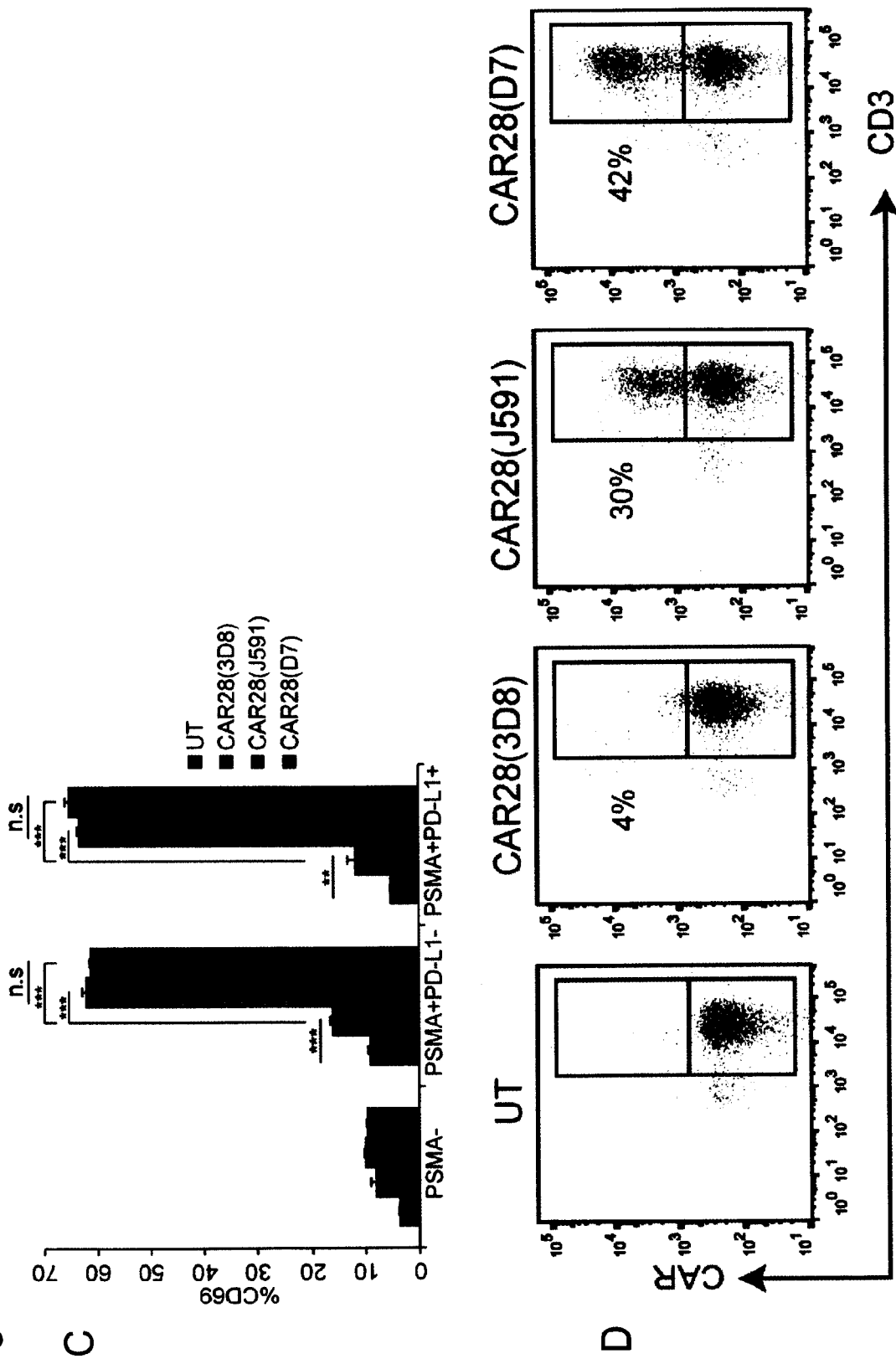


Fig.15

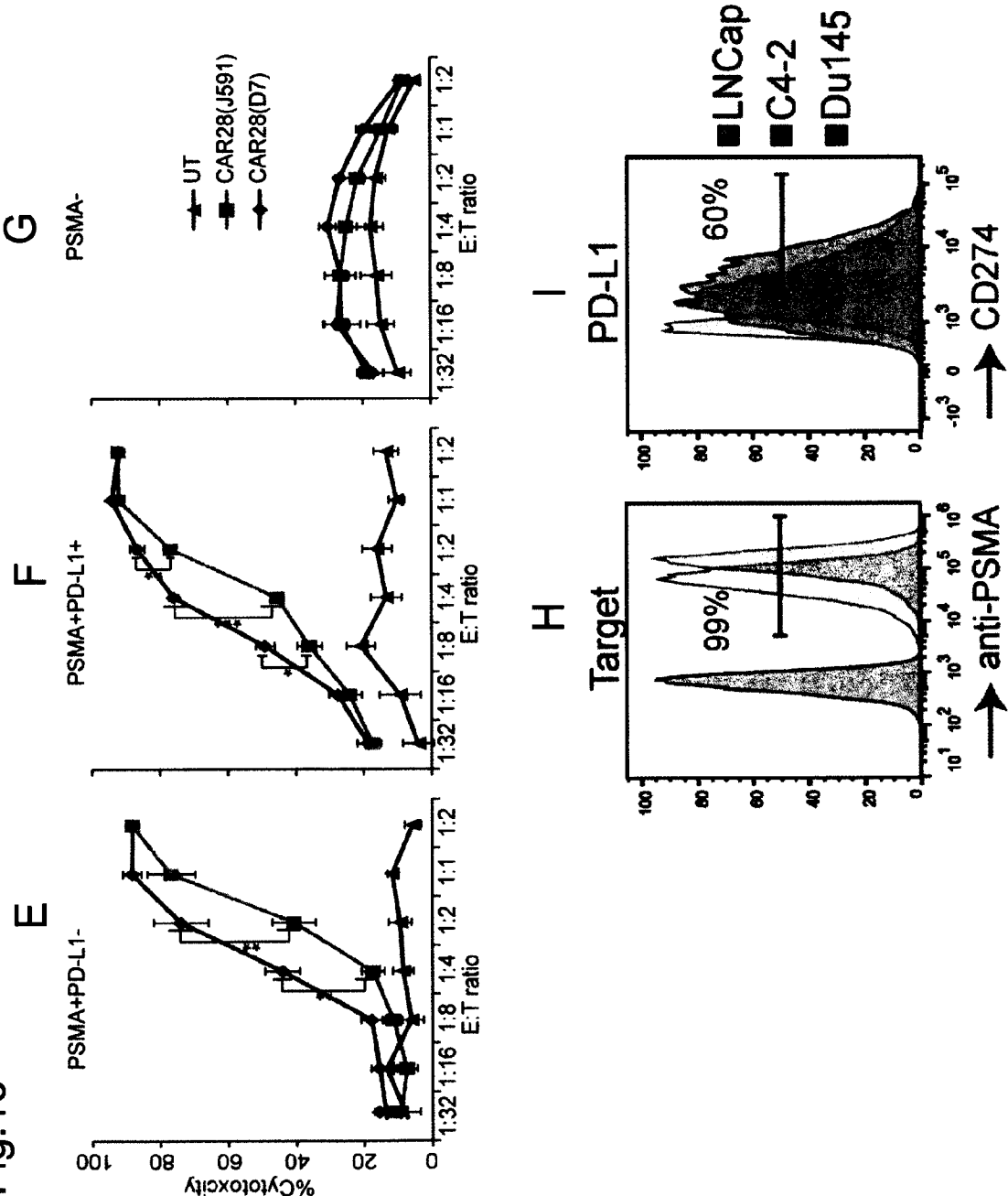
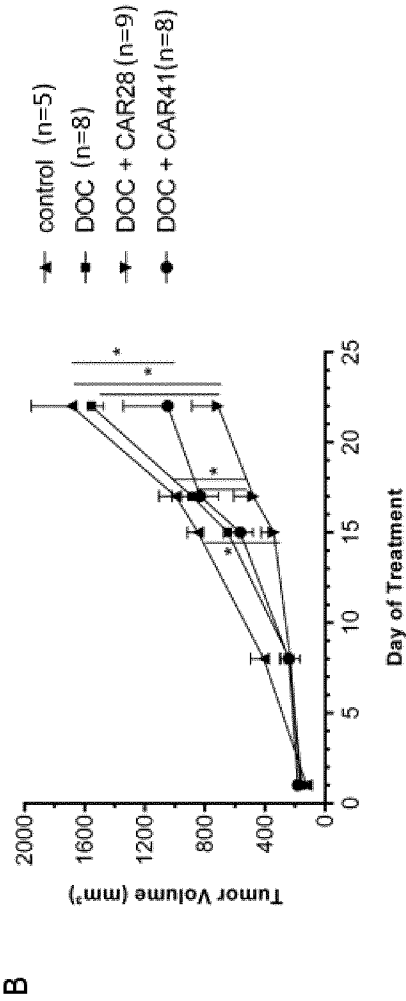
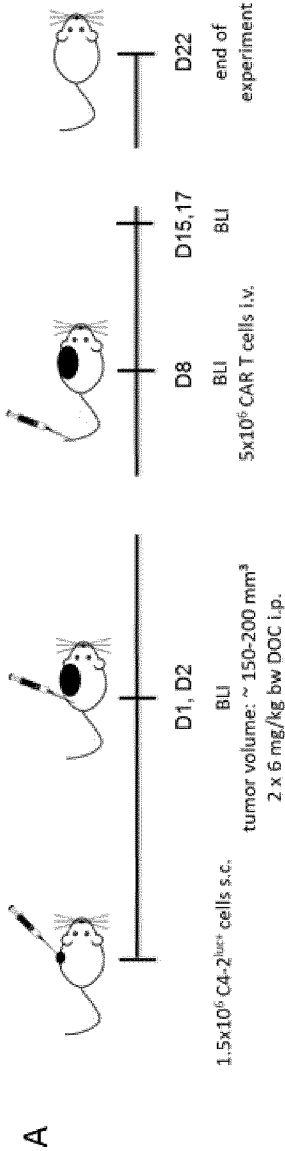


Fig. 16



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/065822

A. CLASSIFICATION OF SUBJECT MATTER		
INV.	C07K16/30 A61K39/00 A61K39/395 A61K31/00 C07K14/725	
ADD.		
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)		
A61K C07K A61P		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
EPO-Internal, BIOSIS		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	ALZUBI J ET AL: "A novel chimeric antigen receptor CAR-T cell approach eliminates prostate cancer in a mouse tumour model", HUMAN GENE THERAPY, vol. 29, no. 12, 1 December 2018 (2018-12-01), page A21, XP002793064, & CONFERENCE ON CHANGING THE FACE OF MODERN MEDICINE - STEM CELL AND GENE THERAPY; LAUSANNE, SWITZERLAND; OCTOBER 16-19, 2018	1-11, 13-15
Y,P	the whole document ----- -/-	12
☒ 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
18 July 2019		30/07/2019
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Bumb, Peter

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2019/065822

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WOLF PHILIPP ET AL: "Preclinical Evaluation of a Recombinant Anti-Prostate Specific Membrane Antigen Single-Chain Immunotoxin Against Prostate Cancer", JOURNAL OF IMMUNOTHERAPY, LIPPINCOTT WILLIAMS & WILKINS, US, vol. 33, no. 3, 31 March 2010 (2010-03-31), pages 262-271, XP009510754, ISSN: 1524-9557, DOI: 10.1097/CJI.0B013E3181C5495C abstract -----	1,2,4-6
A	EP 2 363 486 A1 (UNIVERSITAETSKLINIKUM FREIBURG [DE]) 7 September 2011 (2011-09-07) sequence 22 -----	1,2,4-6
Y	MA QIANGZHONG ET AL: "Advanced generation anti-prostate specific membrane antigen designer T cells for prostate cancer immunotherapy.", THE PROSTATE FEB 2014, vol. 74, no. 3, February 2014 (2014-02), pages 286-296, XP002786351, ISSN: 1097-0045 page 287, right-hand column figure 7B -----	12
Y	JUNGHANS RICHARD P ET AL: "Phase I Trial of Anti-PSMA Designer CAR-T Cells in Prostate Cancer: Possible Role for Interacting Interleukin 2-T Cell Pharmacodynamics as a Determinant of Clinical Response.", THE PROSTATE 10 2016, vol. 76, no. 14, October 2016 (2016-10), pages 1257-1270, XP002786352, ISSN: 1097-0045 page 1259, left-hand column abstract -----	12
Y	MA Q ET AL: "ANTI-PROSTATE SPECIFIC MEMBRANE ANTIGEN DESIGNER T CELLS FOR PROSTATE CANCER THERAPY", PROST, WILEY-LISS, NEW YORK, NY, US, vol. 61, no. 1, 15 September 2004 (2004-09-15), pages 12-25, XP008075302, ISSN: 0270-4137, DOI: 10.1002/PROS.20073 [retrieved on 2004-03-05] figure 2 ----- -/--	12

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2019/065822

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SANTORO STEPHEN P ET AL: "T cells bearing a chimeric antigen receptor against prostate-specific membrane antigen mediate vascular disruption and result in tumor regression.", CANCER IMMUNOLOGY RESEARCH JAN 2015, vol. 3, no. 1, January 2015 (2015-01), pages 68-84, XP055347210, ISSN: 2326-6074 page 69, left-hand column, last paragraph - right-hand column, paragraph first page 69, right-hand column, paragraph second page 75, left-hand column page 75, right-hand column - page 79, left-hand column -----	1-15
A	XIAO-SONG ZHONG ET AL: "Chimeric Antigen Receptors Combining 4-1BB and CD28 Signaling Domains Augment PI3kinase/AKT/Bcl-XL Activation and CD8+ T Cell-mediated Tumor Eradication", MOLECULAR THERAPY, vol. 18, no. 2, 1 February 2010 (2010-02-01), pages 413-420, XP055023623, ISSN: 1525-0016, DOI: 10.1038/mt.2009.210 figures 1, 3 page 419, left-hand column -----	1-15
A	WO 2009/017823 A2 (US GOV HEALTH & HUMAN SERV [US]; NEVILLE DAVID [US]; WOO JUNG-HEE [US]) 5 February 2009 (2009-02-05) sequence 12 page 73 - page 74 -----	1-15

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2019/065822

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2363486	A1	07-09-2011	AT 505544 T 15-04-2011
		AU 2006251445 A1 30-11-2006	
		CA 2609682 A1 30-11-2006	
		CN 101233236 A 30-07-2008	
		CY 1111409 T1 05-08-2015	
		DK 1883698 T3 06-06-2011	
		EP 1726650 A1 29-11-2006	
		EP 1883698 A1 06-02-2008	
		EP 2363486 A1 07-09-2011	
		ES 2362795 T3 13-07-2011	
		JP 5038297 B2 03-10-2012	
		JP 5524270 B2 18-06-2014	
		JP 6110321 B2 05-04-2017	
		JP 2008541711 A 27-11-2008	
		JP 2012197278 A 18-10-2012	
		JP 2014141491 A 07-08-2014	
		JP 2016190839 A 10-11-2016	
		PT 1883698 E 28-06-2011	
		SI 1883698 T1 30-06-2011	
		US 2009041789 A1 12-02-2009	
		US 2012231024 A1 13-09-2012	
		US 2014155583 A1 05-06-2014	
		WO 2006125481 A1 30-11-2006	
WO 2009017823	A2	05-02-2009	AU 2008282729 A1 05-02-2009
			CA 2695382 A1 05-02-2009
			EP 2187971 A2 26-05-2010
			US 2011189209 A1 04-08-2011
			WO 2009017823 A2 05-02-2009