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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
16 March 2017 (16.03.2017)

WIPO | PCT

(10) International Publication Number
WO 2017/041143 A1

(51) International Patent Classification:
C12N 15/62 (2006.01) *C07K 19/00* (2006.01)
C12N 5/0781 (2010.01) *A61K 35/17* (2015.01)
C12N 5/0783 (2010.01) *A61P 35/00* (2006.01)

(21) International Application Number:
PCT/AU2016/050851

(22) International Filing Date:
10 September 2016 (10.09.2016)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
2015903719 11 September 2015 (11.09.2015) AU

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

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

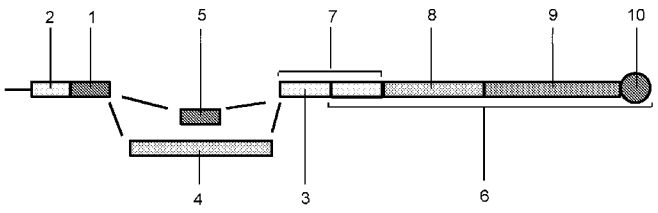
Published:

— *with international search report (Art. 21(3))*

— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

FIGURE 1



(57) Abstract: The present invention relates to chimeric antigen receptors (CARs) directed to cells expressing a dysfunctional or non-functional P2X purinoceptor 7 receptor. Further provided are methods of targeting neoplastic cells and tumours expressing a dysfunctional or non- functional P2X purinoceptor 7 receptor and methods of treating and preventing cancer is a subject.

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CHIMERIC ANTIGEN RECEPTORS AND USES THEREOF

PRIORITY CLAIM

[0001] This application claims priority from Australian provisional patent application number 2015903719 filed on 11 September 2015, the contents of which are to be taken as incorporated herein by this reference.

FIELD OF THE INVENTION

[0002] The present invention relates to chimeric antigen receptors, T cells expressing chimeric antigen receptors and methods of using chimeric antigen receptors for the prevention and/or treatment of cancer.

BACKGROUND OF THE INVENTION

[0003] The immune system has highly evolved and specific mechanisms that protect us from a range of pathologies. Amongst these pathologies is the detection and elimination of unwanted pathogens such as bacterial infections, virally infected cells, and importantly, mutated cells that may cause malignant neoplasia (cancer). The ability for the immune system to prevent the formation and growth of cancers is dependent on the ability of the cells of the immune system to distinguish between a 'healthy' cell and a 'diseased' (e.g. neoplastic or pre-neoplastic) cell. This is achieved by recognition of cell markers (antigens) that are indicative of the transition in a cell from a healthy state to a diseased state.

[0004] There have been many attempts to develop immunotherapeutic approaches to treat cancer by manipulating or directing the immune system to target cells expressing cancer cell antigens. Immunotherapeutic approaches have largely centred on either exploiting the humoral immune system by utilising isolated or engineered antibodies or, more recently, the cellular arm of the immune system.

[0005] Early attempts to utilise cellular immunotherapy for the treatment of cancer utilised T lymphocytes isolated from tumours and expanded *ex vivo*. Whilst this approach has provided some initial promise in early investigations, there are many technical challenges associated with this approach. The ability to isolate and expand T cell populations to clinically relevant numbers is technically challenging and the poorly controlled nature of the expansion results in a final T cell population that is distinctly heterogeneous, and may contain only a small number of cancer antigen-specific T cells. As a result, the efficacy of this method is unpredictable and variable.

[0006] In order to address some of the shortfalls related to the use of *ex vivo* expanded tumour-isolated T cells, chimeric antigen receptors (CARs or artificial T cell receptors) began to be developed in the late 1980s. Chimeric antigen receptors are created by linking an extracellular region that is specific for a desired antigen to a signalling region, resulting in an antigen-specific receptor that can induce T cell function.

[0007] Transformation of isolated T cells with CARs results in a population of T cells that are specific for a given antigen. As a result, large populations of antigen-specific T cells can be generated and used for immunotherapy.

[0008] Initial clinical trials of CAR-transformed T cells specific for tumour associated antigens were promising. However, the efficacy of the CAR-transformed T cells led to significant hypercytokinemia, and ultimately death in some patients. These adverse effects are largely believed to be induced by on-target, but off-tumour activity of the CAR-transformed T cells induced as a result of endogenous expression of the cognate antigen for the CAR on healthy, non-cancerous, cell populations.

[0009] It is therefore apparent that there is a need for the development of a CAR that targets a tumour-associated antigen which is selectively expressed by cancerous cells but not endogenously expressed on non-cancerous cells.

[0010] The discussion of documents, acts, materials, devices, articles and the like is included in this specification solely for the purpose of providing a context for the present invention. It is not suggested or represented that any or all of these matters formed part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed before the priority date of each claim of this application.

SUMMARY OF THE INVENTION

[0011] The present invention is predicated in part on the recognition that due to the significant 'on-target' but 'off-tumour' activity of CAR-expressing immune cells, there is a need for the development of a CAR, and a genetically modified cell expressing the same, which targets a marker specifically associated with a range of neoplastic (cancerous) or pre-neoplastic (pre-cancerous) cells. The inventors have recognised that a dysfunctional $P2X_7$ receptor is a suitable marker for targeting with a CAR.

[0012] Accordingly, in a first aspect, the present invention provides a chimeric antigen receptor including an antigen-recognition domain and a signalling domain, wherein the antigen-recognition domain recognises a dysfunctional $P2X_7$ receptor.

[0013] In some embodiments, the antigen-recognition domain recognises an epitope associated with an adenosine triphosphate (ATP)-binding site of the dysfunctional $P2X_7$ receptor. In some embodiments, the dysfunctional $P2X_7$ receptor has a reduced capacity to bind ATP at the ATP-binding site compared to an ATP-binding capacity of a wild-type (functional) $P2X_7$ receptor. In some embodiments the dysfunctional $P2X_7$ receptor cannot bind ATP at the ATP-binding site.

[0014] In some embodiments, the dysfunctional $P2X_7$ receptor has a conformational change that renders the receptor dysfunctional. In some embodiments, the conformational change is a change of an amino acid from the trans-conformation to the cis-conformation. In some embodiments, the amino acid that has changed from a trans-conformation to a cis-conformation is proline at amino acid position 210 of the dysfunctional $P2X_7$ receptor.

[0015] In some embodiments, the antigen-recognition domain recognises an epitope that includes the proline at amino acid position 210 of the dysfunctional $P2X_7$ receptor. In some embodiments, the antigen-recognition domain recognises an epitope that includes one or more amino acid residues spanning from glycine at amino acid position 200 to cysteine at amino acid position 216, inclusive, of the dysfunctional $P2X_7$ receptor.

[0016] The antigen-recognition domain of the CAR can be any suitable molecule that can interact with and specifically recognise a dysfunctional $P2X_7$ receptor. However, in some embodiments, the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of an antibody, or a fragment thereof, that binds to the dysfunctional $P2X_7$ receptor. In some embodiments, the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a fragment-antigen binding (Fab) portion of an antibody that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the antibody is a humanised antibody.

[0017] In some embodiments, the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a single-chain variable fragment (scFv) or a multivalent scFv that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the multivalent scFv is a di-valent or tri-valent scFv.

[0018] In some embodiments, the antigen-recognition domain includes amino acid sequence homology to a single-antibody domain (sdAb) that binds to a dysfunctional $P2X_7$ receptor.

[0019] In some embodiments, the antigen-recognition domain includes a binding peptide that includes amino acid sequence homology to one or more CDR regions of an antibody that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the biding peptide includes amino acid sequence homology to the CDR1, 2 and 3 domains of the V_H and/or V_L chain of an antibody that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the antigen recognition domain includes one or more amino acid sequences which are at least 50%, 60%, 70%, 80%, 90% or 94% identical to any one of the regions spanning positions 30 to 35, 50 to 67 or 98 to 108 of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34. In some embodiments, the antigen recognition domain includes one or more of the sequences spanning positions 30 to 35, 50 to 67 or 98 to 108 of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34. In some embodiments, the antigen recognition domain includes one or more of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34.

[0020] In some embodiments, the signalling domain includes a portion derived from an activation receptor. In some embodiments, the activation receptor is a member of the CD3 co-receptor complex or is an Fc receptor. In some embodiments, the portion derived from the CD3 co-receptor complex is CD3-ζ. In some embodiments, the portion derived from the Fc receptor is FcεRI or FcγRI.

[0021] In some embodiments, the signalling domain includes a portion derived from a co-stimulatory receptor. In some embodiments, the signalling domain includes a portion derived from an activation receptor and a portion derived from a co-stimulatory receptor. In some embodiments, the co-stimulatory receptor is selected from the group consisting of CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

[0022] In a second aspect, the present invention provides a nucleic acid molecule including a nucleotide sequence encoding the chimeric antigen receptor according to the first aspect of the invention.

[0023] In a third aspect, the present invention provides a nucleic acid construct that includes a nucleic acid molecule according to the second aspect of the invention. In some embodiments, expression of the nucleic acid molecule is under the control of a transcriptional control sequence. In some embodiments, the transcriptional control sequence may be a constitutive promoter or an inducible promoter.

[0024] In some embodiments of the third aspect of the invention, the nucleic acid construct further includes an internal ribosome entry site (IRES) that allows for translation initiation within the mRNA once expressed from the nucleic acid construct.

[0025] In some embodiments of the third aspect of the invention, the nucleic acid construct is a vector such as a viral vector, which can be used to transform a T cell to induce expression of the CAR.

[0026] In a fourth aspect, the present invention provides a genetically modified cell that includes a CAR according to the first aspect of the invention. In some embodiments, the cell includes two or more different CARs.

[0027] In a fifth aspect, the present invention provides a genetically modified cell that includes a nucleic acid molecule according to the second aspect of the invention, or a nucleic acid construct according to the third aspect of the invention, or a genomically integrated form of the construct. In some embodiments, the nucleic acid molecule or the nucleic acid construct encodes two or more different CARs.

[0028] In some embodiments of the fourth and fifth aspects of the invention, the two or more different CARs have different signalling domains.

[0029] In some embodiments of the fourth and fifth aspects of the invention, the cell includes a first CAR with a signalling domain including a portion derived from an activation receptor and a second CAR with a signalling domain including a portion derived from a co-stimulatory receptor. In some embodiments, the activation receptor is a member of the CD3 co-receptor complex or is an Fc receptor. In some embodiments, the co-stimulatory receptor is selected from the group consisting of CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

[0030] In some embodiments of the fourth and fifth aspects of the invention, the cell is further modified so as to constitutively express co-stimulatory receptors. In some embodiments, the cell is further modified so as to express ligands for the co-stimulatory receptors, thereby facilitating auto-stimulation of the cell.

[0031] In some embodiments of the fourth and fifth aspects of the invention, the cell is further modified to secrete cytokines. In some embodiments, the cytokines are selected from the group consisting of IL-2, IL- 7, IL-12, IL-15, IL-17 and IL-21, or a combination thereof.

[0032] In some embodiments of the fourth and fifth aspects of the invention, the cell is a leukocyte. In some embodiments, the cell is a Peripheral Blood Mononuclear Cell (PBMC), a lymphocyte, a T cell (including a CD4+ T cell or a CD8+ T cell), a natural killer cell, or a natural killer T cell.

[0033] In a sixth aspect, the present invention provides a method of killing a cell expressing a dysfunctional $P2X_7$ receptor, the method including exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified cell having a chimeric antigen receptor, wherein the chimeric antigen receptor is directed against a dysfunctional $P2X_7$ receptor.

[0034] In some embodiments of the sixth aspect of the invention, the CAR directly recognises the dysfunctional $P2X_7$ receptor, or recognises the dysfunctional $P2X_7$ receptor via an intermediate. In some embodiments, the intermediate is a probe that binds to a dysfunctional $P2X_7$ receptor and the CAR recognises the probe. In some embodiments, the probe is an antibody or an aptamer. In some embodiments, the probe includes a tag and the CAR recognises the tag.

[0035] In a seventh aspect, the present invention provides a method of killing a cell expressing a dysfunctional P2X7, the method including exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified cell according to the fourth or fifth aspects of the invention.

[0036] In some embodiments of the sixth and seventh aspects of the invention, the cell expressing a dysfunctional $P2X_7$ receptor is exposed to the genetically modified cell together with an exogenous cytokine. In some embodiments, the genetically modified cell is a genetically modified cell, autologous to the cell expressing a dysfunctional $P2X_7$ receptor.

In some embodiments of the sixth and seventh aspects of the invention, the cell expressing a dysfunctional $P2X_7$ receptor is a cancer cell. In some embodiments the cancer is selected from the group consisting of; brain cancer, oesophageal cancer, mouth cancer, tongue cancer, thyroid cancer, lung cancer, stomach cancer, pancreatic cancer, kidney cancer, colon cancer, rectal cancer, prostate cancer, bladder cancer, cervical cancer, epithelial cell cancers, skin cancer, leukaemia, lymphoma, myeloma, breast cancer, ovarian cancer, endometrial

cancer and testicular cancer. In some embodiments the cancer is selected from the group consisting of; lung cancer, oesophageal cancer, stomach cancer, colon cancer, prostate cancer, bladder cancer, cervical cancer, vaginal cancers, epithelial cell cancers, skin cancer, blood-related cancers, breast cancer, endometrial cancer, uterine cancer and testicular cancer.

[0037] In some embodiments of the sixth and seventh aspects of the invention, the cancer is metastatic. In some embodiments, the cancer is stage III cancer or is stage IV cancer.

[0038] In an eighth aspect, the present invention provides a method of expanding *in vitro* the genetically modified cell according to the fourth or fifth aspects of the invention, the method including the step of exposing the cell to an antigen for the CAR. In some embodiments, the method includes the further step of exposing the cell to a cytokine.

[0039] In a ninth aspect, the present invention provides a method of expanding *in vitro* the genetically modified cell according to the fourth or fifth aspects of the invention, the method including the step of exposing the cell to an antigen for the CAR and simultaneously exposing the cell to a cytokine.

[0040] In some embodiments of the eighth and ninth aspects of the invention, the cytokine is a member of the IL-2 subfamily, the interferon subfamily, the IL-10 subfamily, the IL-1 subfamily, the IL-17 subfamily or the TGF-β subfamily.

[0041] In some embodiments of the eighth and ninth aspects of the invention, the cytokine is selected from the group consisting of IFN-γ, IL-2, IL-5, IL- 7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, TNF-α, TGF-β1, TGF-β2, TGF-β3 and GM-CSF, or a combination thereof.

[0042] In a tenth aspect, the present invention provides a method of expanding *in vitro* the genetically modified cell according to the fourth or fifth aspects of the invention, the method including exposing the cell to immobilised anti-CD3 and anti-CD28 antibodies. In some embodiments of the tenth aspect of the invention, the antibodies are immobilised on a beaded substrate (for example on "Human Activator" Dynabeads™). In some embodiments of the tenth aspect of the invention, the antibodies are immobilised on a surface of a tissue culture vessel such as a surface of a culture flask, plate or bioreactor.

[0043] In an eleventh aspect, the present invention provides a pharmaceutical composition including a genetically modified cell according to the fourth or fifth aspects of the invention

and a pharmaceutically acceptable carrier. In some embodiments, the pharmaceutical composition includes suitable adjuvants which may consist of cytokines. In some embodiments, the pharmaceutical composition may also include an intermediate as described herein.

BRIEF DESCRIPTION OF THE FIGURES

[0044] For a further understanding of the aspects and advantages of the present invention, reference should be made to the following detailed description, taken in conjunction with the accompanying drawings.

[0045] FIGURE 1 – a schematic showing the arrangement of an anti-non-functional (nf) $P2X_7$ receptor chimeric antigen receptor (CAR) according to an embodiment of the present invention.

[0046] FIGURE 2 – a schematic showing the BLIV plasmid used for expression of the anti-nf P2X7 receptor CAR in Figure 1.

[0047] FIGURE 3 – an electrophoresis gel showing restriction fragments from *Bam*HI restricted DNA isolated from *E.coli* clones transformed with the BLIV plasmid.

[0048] FIGURE 4 – an electrophoresis gel showing restriction fragments from *Eco*RI, *Bam*HI and *Pst*I restricted DNA isolated from selected *E.coli* clones transformed with the BLIV plasmid.

[0049] FIGURE 5 – shows microscopy images of 293T cells transfected with plasmids required for the construction of lentiviral vectors containing the BLIV-CAR-short hinge construct and 293T cells transduced with supernatant containing the lentiviral vectors.

[0050] FIGURE 6 – shows microscopy images of 293T cells transfected with plasmids required for the construction of lentiviral vectors containing the BLIV-CAR-long hinge construct and 293T cells transduced with supernatant containing the lentiviral vectors.

[0051] FIGURE 7 – FACS analysis of the cell purity of T cells purified with the RosetteSep human CD8+ T cell enrichment kit.

[0052] FIGURE 8 – FACS analysis of killing assays comprising the co-culturing of CD8+ T cells and BT549 cells.

[0053] FIGURE 9 – Graph illustrating the percentage of dye-labelled target cells deleted after 48hr of co-culture with CD8+ T cells transduced with lentiviral vectors containing the BLIV-CAR-short hinge and BLIV-CAR-short hinge plasmids compared to non-transduced CD8+ T cells and CD8+ T cells transduced with empty BLIV plasmids.

[0054] FIGURE 10 – Alignment of the PEP2-2-1-1, PEP2-472-2 and PEP2-2-12 binding peptides with antibodies directed against the nf-$P2X_7$ receptor.

[0055] FIGURE 11 – A schematic showing the arrangement of an anti-nf $P2X_7$ receptor CAR according to a further embodiment of the present invention.

[0056] FIGURE 12 – A schematic showing the pCDH plasmid used for expression of an anti-nf $P2X_7$ receptor CAR of Figure 11.

[0057] FIGURE 13 – An electrophoresis gel showing restriction fragments from *Eco*RI and *Not* I restricted DNA isolated from selected Sure 2 clones, transformed with the pCDH plasmid.

[0058] FIGURE 14 – FACS analysis of the efficiency of transfection of HEK293T cells.

[0059] FIGURE 15 – Representative histograms of FACS analysis of lentiviral transduction efficiency.

[0060] FIGURE 16 – FACS analysis of the percentage of transduced CD8 cells expressing GFP.

[0061] FIGURE 17 – Illustration of the backbone of the fusion protein for generation of non-functional and functional $P2X_7$ receptors.

[0062] FIGURE 18 – An electrophoresis gel showing restriction fragments from *Bam HI* and *PmeI* restricted DNA isolated from selected *E.cloni*®10G clones transformed with EXD2_K193A or EXD2_WT containing pDONR-107 vectors.

[0063] FIGURE 19 – An electrophoresis gel showing restriction fragments from *Bam HI* restricted DNA isolated from selected *E.cloni*®10G clones transformed with EXD2_K193A or EXD2_WT containing pLV-416 vectors.

[0064] FIGURE 20 – FACS analysis of transduction of lentiviral packaging of HEK293 cells with pLV-416- EXD2_K193A and pLV-416-EXD2_WT.

[0065] FIGURE 21 – FACS analysis of transduction of HEK293 with lentivirus containing either pLV-416- EXD2_K193A or pLV-416-EXD2_WT constructs.

[0066] FIGURE 22 – Graph illustrating the killing of nfP2X$_7$ expressing HEK target cells and 231 breast cancer cell by T cells expressing PEP2-2-1-1, PEP2-472-2 CARs

DETAILED DESCRIPTION OF THE INVENTION

[0067] The nucleotide and polypeptide sequences referred to herein are represented by a sequence identifier number (SEQ ID NO:). A summary of the sequence identifiers is provided in Table 1. A sequence listing is also provided at the end of the specification.

TABLE 1

Summary of Sequence Identifiers

Sequence Identifier	Sequence
SEQ ID NO: 1	Human P2X$_7$ receptor mRNA sequence
SEQ ID NO: 2	Human P2X$_7$ receptor coding (cDNA) sequence
SEQ ID NO: 3	Human P2X$_7$ receptor amino acid sequence
SEQ ID NO: 4	CD3ζ chain amino acid sequence
SEQ ID NO: 5	CD3ε chain amino acid sequence
SEQ ID NO: 6	CD3γ chain amino acid sequence
SEQ ID NO: 7	CD3δ chain amino acid sequence
SEQ ID NO: 8	FcεR1 amino acid sequence
SEQ ID NO: 9	FcγRI amino acid sequence
SEQ ID NO: 10	PEP2-2-3 amino acid sequence
SEQ ID NO: 11	PEP2-2-3 nucleotide sequence
SEQ ID NO: 12	CD8a signalling amino acid sequence
SEQ ID NO: 13	CD8a signalling nucleotide sequence
SEQ ID NO: 14	Long hinge amino acid sequence
SEQ ID NO: 15	Long hinge nucleotide sequence
SEQ ID NO: 16	Short hinge amino acid sequence
SEQ ID NO: 17	Short hinge nucleotide sequence

Sequence Identifier	Sequence
SEQ ID NO: 18	Amino acid sequence of a portion of the CD28 co-stimulatory receptor
SEQ ID NO: 19	Nucleotide sequence coding for SEQ ID NO: 18
SEQ ID NO: 20	Amino acid sequence of a portion of the OX40 co-stimulatory receptor
SEQ ID NO: 21	Nucleotide sequence coding for SEQ ID NO: 20
SEQ ID NO: 22	Amino acid sequence of a portion of the CD3 zeta co-receptor complex
SEQ ID NO: 23	Nucleotide sequence coding for SEQ ID NO: 22
SEQ ID NO: 24	P2A amino acid sequence
SEQ ID NO: 25	P2A nucleotide sequence
SEQ ID NO: 26	PEP2-2-3 binding peptide CAR amino acid sequence – long hinge
SEQ ID NO: 27	PEP2-2-3 binding peptide CAR amino acid sequence – short hinge
SEQ ID NO: 28	PEP2-2-3 binding peptide CAR nucleotide sequence – long hinge
SEQ ID NO: 29	PEP2-2-3 binding peptide CAR nucleotide sequence – short hinge
SEQ ID NO: 30	Human CD8 leader amino acid sequence
SEQ ID NO: 31	Human CD8 nucleotide sequence
SEQ ID NO: 32	Amino acid sequence of PEP2-2-1-1 binding peptide
SEQ ID NO: 33	Amino acid sequence of PEP2-472-2 binding peptide
SEQ ID NO: 34	Amino acid sequence of PEP2-2-12 binding peptide
SEQ ID NO: 35	Nucleotide sequence of PEP2-2-1-1 CAR
SEQ ID NO: 36	Nucleotide sequence of PEP2-472-2 CAR
SEQ ID NO: 37	Nucleotide sequence of PEP2-2-12 CAR
SEQ ID NO: 38	pCHD-CMV-For primer
SEQ ID NO: 39	pCHD-coGFP-Rev primer
SEQ ID NO: 40	2-2-1-1-Rev primer
SEQ ID NO: 41	2-2-1-1-For primer
SEQ ID NO: 42	2-472-2-Rev primer
SEQ ID NO: 43	2-472-2-For primer
SEQ ID NO: 44	2-12-2-Rev primer
SEQ ID NO: 45	Com-For-1 primer
SEQ ID NO: 46	Com-For-2 primer
SEQ ID NO: 47	EXD2_K193A gene block
SEQ ID NO: 48	EXD2_WT gene block
SEQ ID NO: 49	EXD-F1 primer
SEQ ID NO: 50	EXD2-R1 primer

Sequence Identifier	Sequence
SEQ ID NO: 51	EXD2-F1 primer
SEQ ID NO: 52	Amino acid sequence of PEP2-2-1-1 CAR
SEQ ID NO: 53	Amino acid sequence of PEP2-472-2 CAR
SEQ ID NO: 54	Amino acid sequence of PEP2-2-12 CAR

[0068] The inventors have recognized that due to the significant 'on-target' but 'off-tumour' activity of chimeric antigen receptor (CAR) expressing immune cells, there is a need for the development of a CAR, and a genetically modified cell expressing the same, which targets a marker specifically associated with neoplastic (cancerous) or pre-neoplastic (pre-cancerous) cells. The inventors have recognised that a dysfunctional $P2X_7$ receptor is a suitable marker for targeting with a CAR expressing immune cell, in a range of cancers.

[0069] Accordingly, in a first aspect, the present invention provides a chimeric antigen receptor (CAR) which includes an antigen-recognition domain and a signalling domain, wherein the antigen-recognition domain recognises a dysfunctional $P2X_7$ receptor.

[0070] Chimeric antigen receptors are artificially constructed proteins that upon expression on the surface of a cell can induce an antigen-specific cellular response. A CAR includes at least two domains; the first domain being an antigen-recognition domain that specifically recognises an antigen, or more specifically an epitope portion, or portions, of an antigen; and the second domain being a signalling domain that is capable of inducing, or participating in the induction, of an intracellular signalling pathway.

[0071] The combination of these two domains determines the antigen specificity of the CAR and the ability of the CAR to induce a desired cellular response, the latter of which is also dependent on the host cell of the CAR. For example, the activation of a CAR expressed in a T-helper cell and having a signalling domain comprising a CD3 activation domain, may, once activated by encountering its cognate antigen, induce the CD4+ T-helper cell to secrete a range of cytokines. In a further example, the same CAR when expressed in a CD8+ cytotoxic T cell, once activated by a cell expressing the cognate antigen, may induce the release of cytotoxins that ultimately lead to the induction of apoptosis of the antigen-expressing cell.

[0072] In addition to the antigen-recognition domain and the signalling domain a CAR may further include additional components, or portions. For example, the CAR may include a transmembrane domain which may comprise a portion of, or may be associated with, the

signalling domain of the CAR. The transmembrane domain is typically one or more hydrophobic helices, which spans the lipid bilayer of a cell and embeds the CAR within the cell membrane. The transmembrane domain of the CAR can be one determinant in the expression pattern of the CAR when associated with a cell. For example, using a transmembrane domain associated with a CD3 co-receptor can permit expression of the CAR in naïve T cells, whilst use of a transmembrane domain from a CD4 co-receptor may direct expression of a CAR in T-helper cells but not cytotoxic T cells.

[0073] A further component or portion of a CAR may be a linker domain. The linker domain (also known as the spacer or hinge domain) may span from the extracellular side of the transmembrane domain to the antigen-recognition domain, thereby linking the antigen-recognition domain to the transmembrane domain. Whilst in some cases a linker domain is not required for a functional CAR (i.e. the antigen-recognition domain can be connected directly to the transmembrane domain) in some circumstances the use of a linker domain allows for greater efficacy of the CAR. The linker domain can have a variety of functions including allowing flexibility of the CAR to permit the necessary orientation of the antigen-recognition domain of the CAR for binding to an antigen. Consequently, the linker domain can be any amino acid sequence that performs this function. One non-limiting example of a linker domain is a domain having amino acid sequence homology to the hinge region of an IgG antibody, such as the IgG1 hinge region. Alternative examples include amino acid sequences having sequence homology to the CH_2CH_3 region of an antibody or portions of the CD3 co-receptor complex, the CD4 co-receptor or the CD8 co-receptor.

[0074] The $P2X_7$ receptor (purinergic receptor P2X, ligand-gated ion channel, 7) is an ATP-gated ion channel that is expressed in a number of species including humans. The receptor is encoded by a gene, the official symbol of which is represented by *P2RX7*. The gene has also been referred to as P2X purinoceptor 7, ATP receptor, P2Z receptor, P2X7 receptor, and purinergic receptor P2X7 variant A. For the purposes of the present disclosure, the gene and encoded receptor will be referred to herein as *P2X7* and $P2X_7$, respectively.

[0075] The mRNA, coding (cDNA), and amino acid sequences of the human *P2X7* gene are set out in SEQ ID NOs: 1 to 3, respectively. The mRNA and amino acid sequences of the human *P2X7* gene are also represented by GenBank Accession Numbers NM_002562.5 and NP_002553.3, respectively. The *P2X7* gene is conserved in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, pig, chicken, zebrafish, and frog. Further details of the *P2X7* gene in human and other species may be accessed from the GenBank database at the National Centre for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). For example, the Gene

ID number for human *P2X7* is 5027, for chimpanzee is 452318, for monkey is 699455, for dog is 448778, for cow is 286814, for mouse is 18439, for zebrafish is 387298, and for frog is 398286. Furthermore, at least 73 organisms have orthologs with the human *P2X7* gene.

[0076] Further details regarding the *P2X7* gene in human and other species can also be found at the UniGene portal of the NCBI (for example see UniGene Hs. 729169 for human $P2X_7$-
http://www.ncbi.nlm.nih.gov/UniGene/clust.cgi?UGID=4540770&TAXID=9606&SEARCH). Alternatively, details of the nucleotide and amino acid sequences for the *P2X7* gene can be accessed from the UniProt database (www.uniprot.org) wherein the UniProt ID for the human *P2X7* gene is Q99572. The contents of the GenBank and UniProt records are incorporated herein by reference.

[0077] The $P2X_7$ receptor is formed from three protein subunits (monomers), wherein in the native receptor in humans at least one of the monomers has an amino acid sequence set forth in SEQ ID NO: 3. It is to be understood that a "$P2X_7$ receptor" as referred to herein also includes naturally occurring variations of the receptor including splice variants, naturally occurring truncated forms and allelic variants of the receptor. A $P2X_7$ receptor may also include subunits that have a modified amino acid sequence, for example those including truncations, amino acid deletions or modifications of the amino acid set forth in SEQ ID NO: 3.

[0078] A "variant" of the *P2X7* gene or encoded protein may exhibit a nucleic acid or an amino acid sequence, respectively, that is at least 80% identical, at least 90% identical, at least 95% identical, at least 98% identical, at least 99% identical, or at least 99.9% identical to a native $P2X_7$ receptor, for example.

[0079] The $P2X_7$ receptor is activated by the binding of ATP to the ATP-binding site of the receptor. This leads to the rapid opening (within milliseconds) of a channel that selectively allows for movement of small cations across the membrane. After a short period of time (within seconds) a large pore is formed in the membrane of a cell that allows for permeation of the cell membrane by molecules up to 900 Da in size. This pore formation ultimately leads to depolarization of the cell and in many cases cytotoxicity and cell death. This role leads to a belief that the $P2X_7$ receptor is involved in apoptosis in a variety of cell types.

[0080] Like other molecules involved in apoptosis, such a Bcl2 and Bax, a decrease or loss in function of the $P2X_7$ receptor can lead to a cell that is comparatively resistant to induced

apoptosis. In many cases this resistance to apoptosis is critical in the transition of a normal 'healthy' cell to a mutated pre-cancerous or cancerous cell. Consequently, the ability to target cells that have a decreased function, or a loss of function, of the $P2X_7$ receptor provides a promising target for cancer therapy.

[0081] Accordingly, in the first aspect of the invention the CAR recognises a dysfunctional $P2X_7$ receptor. As used throughout the specification the term "dysfunctional" with reference to the $P2X_7$ receptor includes a decrease in function of the receptor with respect to its comparative function in a normal non-tumour cell. In some embodiments, the function of $P2X_7$ receptor may be decreased by at least 1%, 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or greater than 99%. In some embodiments, the term "dysfunctional" may include a $P2X_7$ receptor that is non-functional. That is to say that the $P2X_7$ receptor is unable to be induced to permit permeability of cations and other molecules across the cell membrane.

[0082] Any change in the wild-type or native form of the $P2X_7$ receptor that leads to a dysfunctional receptor is encompassed herein. For example, the dysfunctional receptor may be the result of a mutation or alteration in one or more amino acids of the receptor that are associated with ATP binding to the receptor. In effect, the $P2X_7$ receptor is dysfunctional as it has a reduced capacity to, or cannot, bind ATP at the ATP-binding site. In this instance, the antigen-recognition domain of the chimeric antigen receptor will recognise an epitope of the dysfunctional $P2X_7$ receptor associated with the ATP-binding site. Consequently, in some embodiments of the first aspect of the invention, the antigen-recognition domain of the chimeric antigen receptor recognises an epitope of the dysfunctional $P2X_7$ receptor associated with the ATP-binding site. In some embodiments, the dysfunctional $P2X_7$ receptor has a reduced capacity to bind ATP compared to an ATP-binding capacity of a wild-type (functional) $P2X_7$ receptor. In some embodiments the dysfunctional $P2X_7$ receptor cannot bind ATP.

[0083] An alteration in one or more amino acids of the $P2X_7$ receptor may include a conformational change in one or more amino acids of the receptor. Therefore, in some embodiments of the first aspect of the invention the chimeric antigen receptor binds to a dysfunctional $P2X_7$ receptor having a conformational change that renders the receptor dysfunctional. Specifically, this conformational change may be a change in one or more amino acids of the $P2X_7$ receptor from a trans-conformation to a cis-conformation. In some embodiments, a proline at position 210 of the $P2X_7$ receptor changes from a trans-conformation to a cis-conformation. In this instance, the antigen-recognition domain of the CAR may recognise an epitope that includes proline at amino acid position 210 of the $P2X_7$

receptor. In some embodiments of the first aspect of the present invention, the antigen-recognition domain recognises an epitope that includes one or more amino acids spanning from glycine at amino acid position 200 to cysteine at amino acid position 216 (inclusive) of the dysfunctional $P2X_7$ receptor. In some embodiments of the first aspect of the present invention, the antigen-recognition domain recognises an epitope that includes the proline at position 210 of the dysfunctional $P2X_7$ receptor, and one or more of the amino acid residues spanning from glycine at amino acid position 200 to cysteine at amino acid position 216 (inclusive) of the dysfunctional $P2X_7$ receptor.

[0084] Whilst not wanting to be bound by theory, as a result of the conformational change of the proline at position 210 of the $P2X_7$ receptor, the three-dimensional structure of the receptor may be altered. This alteration in the three-dimensional structure may allow the antigen-recognition domain of the CAR to bind to amino acids, or epitopes, previously inaccessible in the native three-dimensional structure of the $P2X_7$ receptor. Therefore, in some embodiments the CAR recognises one or more epitopes of the $P2X_7$ receptor exposed to the antigen-recognition domain as a result of a trans- to cis-conformational change of the proline at position 210 of SEQ ID NO: 3. These epitopes may include one or more of the amino acids at position 200 to 210, or positions 297 to 306, inclusive, of the $P2X_7$ receptor. Accordingly, in some embodiments of the first aspect of the present invention, the antigen-recognition domain recognises an epitope that includes one or more of the amino acids at positions 200 to 210 and/or 297 to 306 of the $P2X_7$ receptor.

[0085] As used throughout the specification the term “recognises” relates to the ability of the antigen-recognition domain to associate with a dysfunctional $P2X_7$ receptor, a portion thereof, or an epitope thereof. In some embodiments, the antigen-recognition domain may directly bind to the dysfunctional $P2X_7$ receptor, or an epitope thereof. In other embodiments, the antigen-recognition domain may bind to a processed form of the dysfunctional $P2X_7$ receptor. As used in this context the term “processed form” relates to forms of the $P2X_7$ receptor which have been truncated or digested as a result of intracellular processing. Consequently, the recognition of the “processed form” of the dysfunctional $P2X_7$ receptor may be as a result of being presented in association with a major histocompatibility complex (MHC).

[0086] The antigen-recognition domain can be any suitable domain that can recognise a dysfunctional $P2X_7$ receptor, or epitope thereof. As used throughout the specification the term “antigen-recognition domain” refers to the portion of the CAR that provides the specificity of the CAR for the dysfunctional $P2X_7$ receptor. The antigen-recognition domain may be all of, or may merely be part of, the extracellular region of the CAR. Suitable antigen-recognition

domains, include, but are not limited to, polypeptides having sequence homology to the antigen-binding site of an antibody, or fragment thereof, that bind to a dysfunctional $P2X_7$ receptor. Therefore, in some embodiments of the first aspect of the invention, the antigen-recognition domain includes an amino acid sequence having homology to an antibody, or a fragment thereof, that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, a portion of the antigen-recognition domain includes an amino acid sequence having homology to an antibody, or a fragment thereof, that binds to the dysfunctional $P2X_7$ receptor. The source homologous antibody sequence can be any suitable sequence of an antibody that has an affinity for the $P2X_7$ receptor. For example the sequence can share sequence homology with an antibody originating from one or more of the following species; human, non-human primate, mouse, rat, rabbit, sheep, goat, ferret, canine, chicken, feline, guinea pig, hamster, horse, cow, or pig. The antigen-recognition domain may share sequence homology with the sequence of a monoclonal antibody produced from a hybridoma cell line. When the originating species of the homologous antibody sequence is not human, the antibody is preferably a humanised antibody. The homologous antibody sequence may also be from a non-mammalian animal species such as a cartilaginous fish (e.g. shark IgNAR antibodies – see WO2012/073048). Alternatively, the antigen binding domain may include a modified protein scaffolds that provide functionality similar to shark antibodies, such as i-bodies which have binding moieties based on shark IgNAR antibodies (see WO2005/118629). Additionally, the antigen-recognition domain could be, could be derived from, or could share sequence homology with any other suitable binding molecule or peptide that can selectively interact with a dysfunctional $P2X_7$ receptor with an affinity sufficient to activate the CAR signalling domain. Methods are known in the art for the identification of antigen-binding proteins such as, *inter alia*, panning phage display libraries, protein affinity chromatography, co-immunoprecipitation and yeast two-hybrid systems (see Srinivasa Rao, V. *et al*. Int J Proteomics, 2014; article ID 147648).

[0087] In some embodiments the antigen-recognition domain of the CAR includes amino acid sequence homology to the amino acid sequence of a fragment-antigen binding (Fab) portion of an antibody that binds to a dysfunctional $P2X_7$ receptor. As will be understood in the art, a Fab portion of an antibody in composed of one constant region and one variable region of each of the heavy and light chains of an antibody. The Fab is the antigen determinant region of the antibody and can be generated by enzymatically cleaving the Fc region from an antibody.

[0088] In some embodiments of the first aspect of the invention, the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a single-

chain variable fragment (scFv) that binds to a dysfunctional $P2X_7$ receptor. As would be understood in the art, a scFv is a fusion protein comprising two portions that may share homology with, or may be identical to, the variable-heavy (VH) and variable-light (VL) chains of an antibody, with the two portions connected together with a linker peptide. For example, the scFv may include VH and VL amino acid sequences that are derived from an antibody that recognises a dysfunctional $P2X_7$ receptor. In this context it will be appreciated that the term "derived from" is not a reference to the source of the polypeptides *per se*, but rather refers to the derivation of the amino acid sequence that constitute a portion of the antigen-binding region. Consequently, the term "derived from" includes synthetically, artificially or otherwise created polypeptides that share sequence identity to an antibody that binds to the dysfunctional $P2X_7$ receptor.

[0089] In some embodiments of the first aspect of the invention, the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a multivalent scFv that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the multivalent scFv is a di-valent or tri-valent scFv.

[0090] In some embodiments of the first aspect of the invention, the antigen-recognition domain has the amino acid sequence of a single-antibody domain (sdAb) that binds to a dysfunctional $P2X_7$ receptor.

[0091] In some embodiments, the antigen-recognition domain includes the amino acid sequence set forth in SEQ ID NO: 10, SEQ ID NO: 32, SEQ ID NO: 33 or SEQ ID NO: 34 or a functional variant thereof.

[0092] In some embodiments, the antigen-recognition domain includes a binding peptide that includes amino acid sequence homology with one or more CDR regions of an antibody that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the biding peptide includes one or more regions having sequence homology with the CDR1, 2 and 3 domains of the V_H and/or V_L chain of an antibody that binds to a dysfunctional $P2X_7$ receptor. In some embodiments, the antigen recognition domain includes one or more sequences which are at least 50%, 60%, 70%, 80%, 90% or 94% identical to any one of the CDR regions spanning positions 30 to 35, 50 to 67 or 98 to 108 of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34. In some embodiments, the antigen recognition domain includes one or more of the sequences spanning positions 30 to 35, 50 to 67 or 98 to 108 of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34. The sequences interspacing the CDR regions of the antigen binding peptides set forth in SEQ ID NOS: 10, 32, 33 or 34 can be any suitable sequence that

permits the appropriate formation and conformation of the CDR regions. In some embodiments, the antigen recognition domain includes a sequence 50%, 60%, 70%, 80% or 90%, 95% or 99% identical to one of the sequences set forth in SEQ ID NOS: 10, 32, 33 or 34.

[0093] Antibodies directed against dysfunctional $P2X_7$ receptors, from which suitable amino acid sequences may be derived, and methods for producing such antibodies, have been described in the art (for example WO2001/020155, WO2003/020762, WO2008/043145, WO2008/043146, WO2009/033233, WO2011/020155 and WO2011/075789). Methods for generating polyclonal and monoclonal antibodies for specific epitopes (such as those set forth previously) would be known to a person skilled in the art. By way of summary, a desired epitope (such as a segment of the dysfunctional $P2X_7$ receptor including the proline at position 210) is injected into a suitable host animal in the presence of an appropriate immunogenic carrier protein and an adjuvant. Serum is then collected from the immunized animal and the antibody can be isolated based on its antibody class or its antigen specificity. Following assessment of the suitability and specificity of the purified antibody, the antibody can be further processed to isolate antigen-binding fragments, or sequenced to identify the relevant VH and VL domains. Suitable epitopes for the production of antibodies directed against the dysfunctional $P2X_7$ receptor are known in the art (see WO2008/043146, WO2010/000041 and WO2009/033233 as examples).

[0094] The signalling domain of the CAR can be any suitable domain that is capable of inducing, or participating in the induction of, an intracellular signalling cascade upon activation of the CAR as a result of recognition of an antigen by the antigen-recognition domain of the CAR. The signalling domain of a CAR will be specifically chosen depending on the cellular outcome desired following activation of the CAR. Whilst there are many possible signalling domains, when used in immunotherapy and cancer therapy the signalling domains can be grouped into two general categories based on the receptor from which they are derived, namely activation receptors and co-stimulatory receptors (see further details below). Therefore, in some embodiments of the first aspect of the invention, the signalling domain includes a portion derived from an activation receptor. In some embodiments, the signalling domain includes a portion derived from a co-stimulatory receptor

[0095] As used throughout the specification the term “portion”, when used with respect to an activation receptor or co-stimulatory receptor, relates to any segment of the receptor that includes a sequence responsible for, or involved in, the initiation/induction of an intracellular signalling cascade following interaction of the receptor with its cognate antigen or ligand. An

example of the initiation/induction of an intracellular signalling cascade for the T cell receptor (TCR) via CD3 is outlined below.

[0096] Whilst not wishing to be bound by theory, the extracellular portion of the TCR largely comprises heterodimers of either the clonotypic TCRα and TCRβ chains (the TCRα/β receptor) or the TCRγ and TCRδ chains (the TCRγδ receptor). These TCR heterodimers generally lack inherent signalling transduction capabilities and therefore they are non-covalently associated with multiple signal transducing subunits of CD3 (primarily CD3-zeta, -gamma, -delta, and -epsilon). Each of the gamma, delta, and epsilon chains of CD3 has an intracellular (cytoplasmic) portion that includes a single Immune-receptor-Tyrosine-based-Activation-Motif (ITAM), whilst the CD3-zeta chain includes three tandem ITAMs. Upon engagement of the TCR by its cognate antigen in the presence of MHC, and the association of a requisite co-receptor such as CD4 or CD8, signalling is initiated which results in a tyrosine kinase (namely Lck) phosphorylating the two tyrosine residues within the intracellular ITAM(s) of the CD3 chains. Subsequently, a second tyrosine kinase (ZAP-70 – itself activated by Lck phosphorylation) is recruited to biphosphorylate the ITAMs. As a result, several downstream target proteins are activated which eventually leads to intracellular conformational changes, calcium mobilisation, and actin cytoskeleton re-arrangement that when combined ultimately lead to activation of transcription factors and induction of a T cell immune response.

[0097] As used throughout the specification the term “activation receptor” relates to receptors, or co-receptors that form a component of, or are involved in the formation of, the T cell receptor (TCR) complex, or receptors involved in the specific activation of immune cells as a result of recognition of an antigenic or other immunogenic stimuli.

[0098] Non-limiting examples of such activation receptors include components of the T cell receptor-CD3 complex (CD3-zeta, -gamma, -delta, and -epsilon), the CD4 co-receptor, the CD8 co-receptor, FC receptors or Natural Killer (NK) cell associated activation receptors such a LY-49 (KLRA1), natural cytotoxicity receptors (NCR, preferably NKp46, NKp44, NKp30 or NKG2 or the CD94/NKG2 heterodimer). Consequently, in some embodiments of the first aspect of the present invention, the signalling domain includes a portion derived from any one or more of a member of the CD3 co-receptor complex (preferably the CD3-ζ chain or a portion thereof), the CD4 co-receptor, the CD8 co-receptor, a Fc Receptor (FcR) (preferably the FcεRI or FcγRI) or NK associated receptors such a LY-49.

[0099] The specific intracellular signal transduction portion of each of the CD3 chains are known in the art. By way of example, the intracellular cytoplasmic region of the CD3ζ chain spans from amino acid 52 to amino acid 164 of the sequence set forth in SEQ ID NO: 4, with the three ITAM regions spanning amino acids 61 to 89, 100 to 128 and 131 to 159 of SEQ ID NO: 4. Furthermore, the intracellular portion of the CD3ε chain spans amino acids 153 to 207 of the sequence set forth in SEQ ID NO: 5, with the single ITAM region spanning amino acids 178 to 205 of SEQ ID NO: 5. The intracellular portion of CD3γ chain spans amino acids 138 to 182 of the sequence set forth in SEQ ID NO: 6 with the single ITAM region spanning amino acids amino acids 149 to 177 of SEQ ID NO: 6. The intracellular portion of CD3δ spans amino acids 127 to 171 of the sequence set forth in SEQ ID NO: 7 with the single ITAM region spanning amino acids 138 to 166 of SEQ ID NO: 7.

[0100] In some embodiments of the first aspect of the present invention, the signalling domain includes a portion derived from any one of CD3 (preferably the CD3-ζ chain or a portion thereof) or an FC receptor (preferably the FcεRI or FcγRI). In some embodiments, the portion of the CD3-ζ co-receptor complex includes the amino acid sequence set forth in SEQ ID NO: 22, or a functional variant thereof.

[0101] The intracellular portions of the FC receptors are known in the art. For example, the intracellular portions of the FcεR1 span amino acids 1 to 59, 118 to 130 and 201 to 244 of the sequence set forth in SEQ ID NO: 8. Furthermore, the intracellular portion of FcγRI spans the amino acids 314 to 374 of the sequence set forth in SEQ ID NO: 9.

[0102] Various combinations of portions of activation receptors can be utilized to form the transmembrane (TM) and intracellular (IC) portions of the CAR for example the CD3ζ TM and CD3ζ IC (Landmeier S. *et al.* Cancer Res. 2007; 67:8335-43; Guest RD. *et al.*, J Immunother. 2005, 28:203-11; Hombach AA. *et al.* J Immunol. 2007; 178: 4650-7), the CD4 TM and CD3ζ IC (James SE. *et al.* J Immunol. 2008;180:7028-38), the CD8 TM and CD3ζ IC (Patel SD. *et al.* Gene Ther. 1999; 6: 412-9), and the FcεRIγ TM and the FcεRIγ IC (Haynes NM. *et al.* J Immunol. 2001; 166: 182-7; Annenkov AE. *et al.* J Immunol. 1998; 161: 6604-13).

[0103] As used throughout the specification the term "co-stimulatory receptor" relates to receptors or co-receptors that assist in the activation of an immune cell upon antigen specific inducement of an activation receptor. As will be understood, co-stimulatory receptors do not require the presence of antigen and are not antigen specific, but are typically one of two signals, the other being an activation signal, which is required for the induction of an immune cellular response. In the context of an immune response a co-stimulation receptor is typically

activated by the presence of its expressed ligand on the surface of an antigen-presenting cell (APC) such as a dendritic cell or macrophage. With specific regard to T cells, co-stimulation is necessary to lead to cellular activation, proliferation, differentiation and survival (all of which are generally referred to under the umbrella of T cell activation), whilst presentation of an antigen to a T cell in the absence of co-stimulation can lead to anergy, clonal deletion and/or the development of antigen specific tolerance. Importantly, co-stimulatory molecules can inform the T cell response to a simultaneously encountered antigen. Generally, an antigen encountered in the context of a 'positive' co-stimulatory molecule will lead to activation of the T cell and a cellular immune response aimed at eliminating cells expressing that antigen. Whilst an antigen encountered in the context of a 'negative' co-receptor will lead to an induced state of tolerance to the co-encountered antigen.

[0104] Non-limiting examples of T cell co-stimulatory receptors include CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137), ICOS. Specifically, CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137), and ICOS all represent 'positive' co-stimulatory molecules that enhance activation of a T cell response. Accordingly, in some embodiments of the first aspect of the present invention, the signalling domain includes a portion derived from any one or more of CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

[0105] In some embodiments of the first aspect of the present invention, the signalling domain includes a portion derived from the CD28, OX40 or 4-1BB co-stimulatory receptors. In some embodiments, the signalling domain includes a portion of the CD28 co-stimulatory receptor. In some embodiments, the signalling domain includes a portion of the OX40 co-stimulatory receptor. In some embodiments, the portion of the OX40 co-stimulatory receptor includes the amino acid sequence set forth in SEQ ID NO: 20, or a functional variant thereof.

[0106] Various combinations of portions of co-stimulatory receptors can be utilized to form the transmembrane (TM) and intracellular (IC) portions of the CAR. For example the CD8 TM and DAP10 IC or CD8 TM and 4-1BB IC (Marin V. *et al.* Exp Hematol. 2007; 35: 1388-97), the CD28 TM and the CD28 IC (Wilkie S. *et al*. J Immunol. 2008;180: 4901-9; Maher J. *et al*. Nat Biotechnol. 2002; 20: 70-5), and the CD8 TM and the CD28 IC (Marin V. *et al.* Exp Hematol. 2007; 35: 1388-97).

[0107] Sequence information for the above-referenced activation and co-stimulatory receptors is readily accessible in a variety of databases. For example, embodiments of human amino acid, gene and mRNA sequences for these receptors is provided in Table 2.

TABLE 2

Summary of Activation and Co-stimulation Receptor Sequence Information

Receptor Name	Uniprot Ref No.	NCBI Gene ID No.	GeneBank mRNA Ref No.
CD3-zeta	P20963	919	GI:166362721
CD3-gamma	P09693	917	GI:166362738
CD3-delta	P04234	915	GI:98985799
CD3-epsilon	P07766	916	GI:166362733
CD4	P0173	920	GI:303522473
CD8 alpha	P01732	925	GI:225007534
CD8 beta	P01966	926	GI:296010927
FcγRI	P12314	2209	GI:31331
FcεR1	Q01362	2206	GI:219881
Ly-49 (KLRAI)	Q7Z556	10748	GI:33114184
NKp46	O76036	9437	GI:3647268
NKp44	O95944	9436	GI:4493701
NKp30	O14931	259197	GI:5823969
CD94	Q13241	3824	GI:1098616
CD27	P26842	939	GI:180084
CD28	P10747	940	GI:338444
CD30	P28908	943	GI:180095
CD40	P25942	958	GI:29850
DAP10	Q9UBK5	10870	GI:5738198
OX40	P43489	7293	GI:472957
4-1BB (CD137)	Q07011	3604	GI:571320
ICOS	Q9Y6W8	29851	GI:9968295
CTLA-4	P16410	1493	GI:291928
PD-1	Q15116	5133	GI:2149002

[0108] Whilst Table 2 is provided with reference to human activation and co-stimulatory receptors, it would be understood by a person skilled in the art that homologous and orthologous versions of each receptor are present in the majority of mammalian and vertebrate species. Therefore, the above-referenced sequences are only provided as non-limiting examples of receptor sequences that may be included in a CAR of the first aspect of the present invention and homologous and orthologous sequences from any desired species may be used to generate a CAR that is suitable for the given species.

[0109] In some embodiments of the first aspect of the invention, the signalling domain includes a portion derived from an activation receptor and a portion derived from a co-stimulatory receptor. Whilst not wishing to be bound by theory, in this context the recognition of an antigen by the antigen-recognition domain of the CAR will simultaneously induce both an intracellular activation signal and an intracellular co-stimulatory signal. Consequently, this will simulate the presentation of an antigen by an APC expressing co-stimulatory ligand. Alternatively, the CAR could have a signalling domain that includes a portion derived from either an activation receptor or a co-stimulatory receptor. In this alternative form, the CAR will only induce either an activating intracellular signalling cascade or a co-stimulatory intracellular signalling cascade.

[0110] In some embodiments of the first aspect of the invention, the CAR will have a signalling domain that includes a portion derived from a single activation receptor and portions derived from multiple co-stimulatory receptors. In some embodiments, the CAR will have a signalling domain that includes portions derived from multiple activation receptors and a portion derived from a single co-stimulatory receptor. In some embodiments, the CAR will have a signalling domain that includes portions derived from multiple activation receptors and portions derived from multiple co-stimulatory receptors. In some embodiments, the CAR will have a signalling domain that includes a portion derived from a single activation receptor and portions derived from two co-stimulatory receptors. In some embodiments, the CAR will have a signalling domain that includes a portion derived from a single activation receptor and portions derived from three co-stimulatory receptors. In some embodiments, the CAR will have a signalling domain that includes portions derived from two activation receptors, and a portion derived from one co-stimulatory receptor. In some embodiments, the CAR will have a signalling domain that includes portions derived from two activation receptors and portions derived from two co-stimulatory receptors. As will be understood there are further variations of the number of activation receptors and co-stimulatory receptors from which the signalling domain can be derived from, and the above examples are not considered to be limiting on the possible combinations included herein.

[0111] In some embodiments of the first aspect of the invention, the chimeric antigen receptor includes the amino acid sequence set forth in SEQ ID NO: 26,SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54, or a functional variant of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54. In some embodiments, the functional variant includes an amino acid sequence which is at least 80% identical to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54.

[0112] As indicated above, the present invention includes a functional variant of any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54. In the context of the present invention, a "functional variant" may include any amino acid sequence provided it maintains the function of any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54.

[0113] As such, the functional variant may, for example, have one or more amino acid insertions, deletions or substitutions relative to one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54; a mutant form or allelic variant of one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54; an ortholog of one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54; a homeologue of one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54; an analog of one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26,SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54; and the like, provided the functional variant maintains the function of any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54.

[0114] For example with respect to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54 the function of a chimeric antigen receptor comprising SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54 is to recognise a dysfunctional $P2X_7$ receptor without significant recognition of the functional $P2X_7$ receptor, and induce an intracellular signal which results in the activation of a T cell expressing the CAR. As would be understood by a person skilled in the art, variation to portions of the amino acid sequence of the chimeric antigen receptor set forth in SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54 may be made without significant alteration of the recognition of the dysfunctional $P2X_7$ receptor and/or activation of a T cell expressing the CAR. Such variations may include, but are not limited to, variations in the hinge region of the chimeric antigen receptor, variations in the transmembrane domain, and variations in the

portions of the activation receptors and/or co-stimulatory receptors that comprise the intracellular domain of the chimeric antigen receptor.

[0115] As indicated above, the functional variant may comprise individual amino acid substitutions, deletions or insertions relative to one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54. For example, a person skilled in the art will recognise that any amino acid can be substituted with a chemically (functionally) similar amino acid and retain function of the polypeptide. Such conservative amino acid substitutions are well known in the art. The following groups in Table 3 each contain amino acids that are conservative substitutions for one another.

TABLE 3

Exemplary amino acid conservative substitutions

Original Residue	Exemplary Substitutions
Ala (A)	Val (V), Leu (L), Ile (I), Gly (G)
Arg (R)	Lys (K)
Asn (N)	Gln (Q), His (H)
Asp (D)	Glu (E)
Cys (C)	Ser (S)
Gln (Q)	Asn (N), His (H)
Glu (E)	Asp (D)
Gly (G)	Pro (P), Ala (A)
His (H)	Asn (N), Gln (Q)
Ile (I)	Leu (L), Val (V), Ala (A)

Original Residue	Exemplary Substitutions
Leu (L)	Ile (I), Val (V), Met (M), Ala (A), Phe (F)
Lys (K)	Arg (R)
Met (M)	Leu (L), Phe (F)
Phe (F)	Leu (L), Val (V), Alal (A)
Pro (P)	Gly (G)
Ser (S)	Thr (T)
Thr (T)	Ser (S)
Trp (W)	Tyr (Y)
Tyr (Y)	Trp (W), Phe (F)
Val (V)	Ile (I), Leu (L), Met (M), Phe (F), Ala (A)

[0116] Furthermore, if desired, unnatural amino acids or chemical amino acid analogues can be introduced as a substitution or addition into a polypeptide encompassed herein. Such amino acids include, but are not limited to, the D-isomers of the common amino acids, 2,4-diaminobutyric acid, α-amino isobutyric acid, 4-aminobutyric acid, 2-aminobutyric acid, 6-amino hexanoic acid, 2-amino isobutyric acid, 3-amino propionic acid, ornithine, norleucine, norvaline, hydroxyproline, sarcosine, citrulline, homocitrulline, cysteic acid, t-butylglycine, t-butylalanine, phenylglycine, cyclohexylalanine, β-alanine, fluoro-amino acids, designer amino acids such as β-methyl amino acids, Cα-methyl amino acids, Nα-methyl amino acids, and amino acid analogues in general.

[0117] As set out above, a functional variant of any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID

NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54 may comprise an amino acid sequence which is at least 80% identical to any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54. In other embodiments, a functional variant may comprise at least 85% amino acid sequence identity, at least 90% amino acid sequence identity, at least 91% amino acid sequence identity, at least 92% amino acid sequence identity, at least 93% amino acid sequence identity, at least 94% amino acid sequence identity, at least 95% amino acid sequence identity, at least 96% amino acid sequence identity, at least 97% amino acid sequence identity, at least 98% amino acid sequence identity, at least 99% amino acid sequence identity, or at least 99.1%, 99.2%, 99.3%, 99.4%, 99.5%, 99.6%, 99.7%, 99.8%, or 99.9% amino acid sequence identity to any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54.

[0118] When comparing amino acid sequences, the sequences should be compared over a comparison window which is determined by the length of the polypeptide. For example, a comparison window of at least 20 amino acid residues, at least 50 amino acid residues, at least 75 amino acid residues, at least 100 amino acid residues, at least 200 amino acid residues, at least 300 amino acid residues, at least 400 amino acid residues, at least 500 amino acid residues, at least 600 amino acid residues, or over the full length of any one of SEQ ID NO: 10, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 32, SEQ ID NO: 33, SEQ ID NO: 34, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54 is envisaged. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms such as the BLAST family of programs as, for example, disclosed by Altschul *et al.*, 1997, *Nucl. Acids Res.* 25: 3389-3402. Global alignment programs may also be used to align similar sequences of roughly equal size. Examples of global alignment programs include NEEDLE (available at www.ebi.ac.uk/Tools/psa/emboss_needle/) which is part of the EMBOSS package (Rice P *et al.*, 2000, *Trends Genet.*, 16: 276-277), and the GGSEARCH program (available at fasta.bioch.virginia.edu/fasta_www2/fasta_www.cgi?rm=compare&pgm=gnw) which is part of the FASTA package (Pearson W and Lipman D, 1988, *Proc. Natl. Acad. Sci. USA*, 85: 2444-2448). Both of these programs are based on the Needleman-Wunsch algorithm which is used to find the optimum alignment (including gaps) of two sequences along their entire length. A

detailed discussion of sequence analysis can also be found in Unit 19.3 of Ausubel *et al* ("Current Protocols in Molecular Biology" John Wiley & Sons Inc, 1994-1998, Chapter 15, 1998).

[0119] In a second aspect, the present invention provides a nucleic acid molecule including a nucleotide sequence encoding the chimeric antigen receptor according to the first aspect of the invention. In some embodiments, the nucleic acid molecule is a non-naturally occurring nucleic acid molecule.

[0120] In some embodiments of the second aspect of the invention, the nucleic acid molecule includes a nucleotide sequence which encodes the amino acid sequence set forth in SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54, or encodes a functional variant of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54. In some embodiments, the functional variant includes an amino acid sequence which is at least 80% identical to SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 52, SEQ ID NO: 53 or SEQ ID NO: 54.

[0121] The nucleic acid molecule may comprise any polyribonucleotide or polydeoxyribonucleotide, which may be unmodified, or modified, RNA or DNA. For example, the nucleic acid molecule may include single- and/or double-stranded DNA, DNA that is a mixture of single- and double-stranded regions, single- and double-stranded RNA, and RNA that is mixture of single- and double-stranded regions, hybrid molecules comprising DNA and RNA that may be single-stranded or, more typically, double-stranded or a mixture of single- and double-stranded regions. In addition, the nucleic acid molecule may comprise triple-stranded regions comprising RNA or DNA or both RNA and DNA. The nucleic acid molecule may also comprise one or more modified bases or DNA or RNA backbones modified for stability or for other reasons. A variety of modifications can be made to DNA and RNA; thus the term "nucleic acid molecule" embraces chemically, enzymatically, or metabolically modified forms.

[0122] In some embodiments of the second aspect of the invention, the nucleic acid molecule includes the nucleotide sequence set forth in SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37.

[0123] It would be understood by a person skilled in the art that any nucleotide sequence which encodes a chimeric antigen receptor having the amino acid sequence set forth in SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37, or a

functional variant of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37, is contemplated by the present invention. For example, variants of SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37 are contemplated which comprise one or more different nucleic acids to SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37 but which still encode identical amino acid sequences. Because of the degeneracy of the genetic code, a large number of nucleic acids can encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Therefore, at every position in SEQ ID NO: 28, SEQ ID NO: 29, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37 where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Accordingly, every nucleotide sequence herein which encodes a chimeric antigen receptor having the amino acid sequence set forth in SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37, or a functional variant of SEQ ID NO: 26, SEQ ID NO: 27, SEQ ID NO: 35, SEQ ID NO: 36 or SEQ ID NO: 37 also describes every possible silent variation of the nucleotide sequence. One of skill will recognise that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleotide sequence that encodes a polypeptide is implicit in each described sequence.

[0124] In a third aspect, the present invention provides a nucleic acid construct including a nucleic acid molecule according to the second aspect of the invention. The nucleic acid construct may further comprise one or more of: an origin of replication for one or more hosts; a selectable marker gene which is active in one or more hosts; and/or one or more transcriptional control sequences.

[0125] As used herein, the term “selectable marker gene” includes any gene that confers a phenotype on a cell in which it is expressed, to facilitate the identification and/or selection of cells which are transfected or transformed with the construct.

[0126] “Selectable marker genes” include any nucleotide sequences which, when expressed by a cell transformed with the construct, confer a phenotype on the cell that facilitates the identification and/or selection of these transformed cells. A range of nucleotide sequences encoding suitable selectable markers are known in the art (for example Mortesen, RM. and Kingston RE. Curr Protoc Mol Biol, 2009; Unit 9.5). Exemplary nucleotide sequences that encode selectable markers include: Adenosine deaminase (ADA) gene; Cytosine deaminase (CDA) gene; Dihydrofolate reductase (DHFR) gene; Histidinol dehydrogenase (hisD) gene;

Puromycin-N-acetyl transferase (PAC) gene; Thymidine kinase (TK) gene; Xanthine-guanine phosphoribosyltransferase (XGPRT) gene or antibiotic resistance genes such as ampicillin-resistance genes, puromycin-resistance genes, Bleomycin-resistance genes, hygromycin-resistance genes, kanamycin-resistance genes and ampicillin-resistance gene; fluorescent reporter genes such as the green, red, yellow or blue fluorescent protein-encoding genes; and luminescence-based reporter genes such as the luciferase gene, amongst others which permit optical selection of cells using techniques such as Fluorescence-Activated Cell Sorting (FACS).

[0127] Furthermore, it should be noted that the selectable marker gene may be a distinct open reading frame in the construct or may be expressed as a fusion protein with another polypeptide (e.g. the CAR).

[0128] As set out above, the nucleic acid construct may also comprise one or more transcriptional control sequences. The term “transcriptional control sequence” should be understood to include any nucleic acid sequence which effects the transcription of an operably connected nucleic acid. A transcriptional control sequence may include, for example, a leader, polyadenylation sequence, promoter, enhancer or upstream activating sequence, and transcription terminator. Typically, a transcriptional control sequence at least includes a promoter. The term “promoter” as used herein, describes any nucleic acid which confers, activates or enhances expression of a nucleic acid in a cell.

[0129] In some embodiments, at least one transcriptional control sequence is operably connected to the nucleic acid molecule of the second aspect of the invention. For the purposes of the present specification, a transcriptional control sequence is regarded as “operably connected” to a given nucleic acid molecule when the transcriptional control sequence is able to promote, inhibit or otherwise modulate the transcription of the nucleic acid molecule. Therefore, in some embodiments, the nucleic acid molecule is under the control of a transcription control sequence, such as a constitutive promoter or an inducible promoter.

[0130] The "nucleic acid construct" may be in any suitable form, such as in the form of a plasmid, phage, transposon, cosmid, chromosome, vector, etc., which is capable of replication when associated with the proper control elements and which can transfer gene sequences, contained within the construct, between cells. Thus, the term includes cloning and expression vehicles, as well as viral vectors. In some embodiments, the nucleic acid construct is a vector. In some embodiments the vector is a viral vector.

[0131] A promoter may regulate the expression of an operably connected nucleic acid molecule constitutively, or differentially, with respect to the cell, tissue, or organ at which expression occurs. As such, the promoter may include, for example, a constitutive promoter, or an inducible promoter. A "constitutive promoter" is a promoter that is active under most environmental and physiological conditions. An "inducible promoter" is a promoter that is active under specific environmental or physiological conditions. The present invention contemplates the use of any promoter which is active in a cell of interest. As such, a wide array of promoters would be readily ascertained by one of ordinary skill in the art.

[0132] Mammalian constitutive promoters may include, but are not limited to, Simian virus 40 (SV40), cytomegalovirus (CMV), P-actin, Ubiquitin C (UBC), elongation factor-1 alpha (EF1A), phosphoglycerate kinase (PGK) and CMV early enhancer/chicken β actin (CAGG).

[0133] Inducible promoters may include, but are not limited to, chemically inducible promoters and physically inducible promoters. Chemically inducible promoters include promoters which have activity that is regulated by chemical compounds such as alcohols, antibiotics, steroids, metal ions or other compounds. Examples of chemically inducible promoters include: tetracycline regulated promoters (e.g. see US Patent 5,851,796 and US Patent 5,464,758); steroid responsive promoters such as glucocorticoid receptor promoters (e.g. see US Patent 5,512,483), ecdysone receptor promoters (e.g. see US Patent 6,379,945) and the like; and metal-responsive promoters such as metallothionein promoters (e.g. see US Patent 4,940,661, US Patent 4,579,821 and US 4,601,978) amongst others.

[0134] As mentioned above, the control sequences may also include a terminator. The term "terminator" refers to a DNA sequence at the end of a transcriptional unit which signals termination of transcription. Terminators are 3'-non-translated DNA sequences generally containing a polyadenylation signal, which facilitate the addition of polyadenylate sequences to the 3'-end of a primary transcript. As with promoter sequences, the terminator may be any terminator sequence which is operable in the cells, tissues or organs in which it is intended to be used. Suitable terminators would be known to a person skilled in the art.

[0135] As will be understood, the nucleic acid construct of the third aspect of the invention can further include additional sequences, for example sequences that permit enhanced expression, cytoplasmic or membrane transportation, and location signals. Specific non-limiting examples include an Internal Ribosome Entry Site (IRES).

[0136] The present invention extends to all genetic constructs essentially as described herein. These constructs may further include nucleotide sequences intended for the maintenance and/or replication of the genetic construct in eukaryotes and/or the integration of the genetic construct or a part thereof into the genome of a eukaryotic cell.

[0137] Methods are known in the art for the deliberate introduction (transfection/transduction) of exogenous genetic material, such as the nucleic acid construct of the third aspect of the present invention, into eukaryotic cells. As will be understood the method best suited for introducing the nucleic acid construct into the desired host cell is dependent on many factors, such as the size of the nucleic acid construct, the type of host cell the desired rate of efficiency of the transfection/transduction and the final desired, or required, viability of the transfected/transduced cells. Non-limiting examples of such methods include; chemical transfection with chemicals such as cationic polymers, calcium phosphate, or structures such as liposomes and dendrimers; non-chemical methods such as electroporation, sonoporations, heat-shock or optical transfection; particle-based methods such as 'gene gun' delivery, magnetofection, or impalefection or viral transduction.

[0138] The nucleic acid construct will be selected depending on the desired method of transfection/transduction. In some embodiments of the third aspect of the invention, the nucleic acid construct is a viral vector, and the method for introducing the nucleic acid construct into a host cell is viral transduction. Methods are known in the art for utilising viral transduction to elicit expression of a CAR in a PBMC (Parker, LL. *et al*. Hum Gene Ther. 2000;11: 2377-87) and more generally utilising retroviral systems for transduction of mammalian cells (Cepko, C. and Pear, W. Curr Protoc Mol Biol. 2001, unit 9.9). In other embodiments, the nucleic acid construct is a plasmid, a cosmid, an artificial chromosome or the like, and can be transfected into the cell by any suitable method known in the art.

[0139] In a fourth aspect the present invention provides a genetically modified cell that includes the chimeric antigen receptor according to the first aspect of the invention.

[0140] In some embodiments of the fourth aspect of the invention, the genetically modified cell includes two or more different CARs.

[0141] In a fifth aspect the invention provides a genetically modified cell that includes the nucleic acid molecule according to the second aspect of the invention, or includes the nucleic acid construct according to the third aspect of the invention, or a genomically integrated form of the nucleic acid construct.

[0142] In some embodiments of the fifth aspect of the invention, the genetically modified cell includes a nucleic acid molecule, or a nucleic acid construct, that encodes for two or more different CARs. In some embodiments of the fifth aspect of the invention, the genetically modified cell includes two or more nucleic acid molecules, or two or more nucleic acid constructs, each of which encodes for a different CAR.

[0143] As referred to herein, a "genetically modified cell" includes any cell comprising a non-naturally occurring and/or introduced nucleic acid molecule or nucleic acid construct encompassed by the present invention. The introduced nucleic acid molecule or nucleic acid construct may be maintained in the cell as a discreet DNA molecule, or it may be integrated into the genomic DNA of the cell.

[0144] Genomic DNA of a cell should be understood in its broadest context to include any and all endogenous DNA that makes up the genetic complement of a cell. As such, the genomic DNA of a cell should be understood to include chromosomes, mitochondrial DNA and the like. As such, the term "genomically integrated" contemplates chromosomal integration, mitochondrial DNA integration, and the like. The "genomically integrated form" of the construct may be all or part of the construct. However, in some embodiments the genomically integrated form of the construct at least includes the nucleic acid molecule of the second aspect of the invention.

[0145] As used herein, the term “different CARs” or “different chimeric antigen receptors” refers to any two or more CARs that have either non-identical antigen-recognition and/or non-identical signalling domains. In one example, “different CARs” includes two CARs with the same antigen-recognition domains (e.g. both CARs may recognise a dysfunctional $P2X_7$ receptor), but have different signalling domains, such as one CAR having a signalling domain with a portion of an activation receptor and the other CAR having a signalling domain with a portion of an co-stimulatory receptor. As will be understood, at least one of the two or more CARs within this embodiment will have an antigen-recognition domain that recognises the dysfunctional $P2X_7$ receptor and the other CAR(s) may take any suitable form and may be directed against any suitable antigen.

[0146] Accordingly, in some embodiments of the fourth and fifth aspects of the invention the two or more different CARs have different signalling domains, and may have identical, or different, antigen-recognition domains. Specifically, the genetically modified cell according to the fourth or fifth aspects of the invention may include a first chimeric antigen receptor with a

signalling domain that includes a portion derived from an activation receptor and a second chimeric antigen receptor with a signalling domain including a portion derived from a co-stimulatory receptor.

[0147] In some embodiments of the fourth or fifth aspects of the invention, the activation receptor (from which a portion of signalling domain is derived) is the CD3 co-receptor complex or is an Fc receptor.

[0148] In some embodiments of the fourth or fifth aspects of the invention, the co-stimulatory receptor (from which a portion of signalling domain is derived) is selected from the group consisting of CD27, CD28, CD-30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

[0149] In some embodiments of the fourth or fifth aspects of the invention, the co-stimulatory receptor (from which a portion of signalling domain is derived) is selected from the group consisting of CD28, OX40 or 4-1BB.

[0150] In some embodiments of the fourth and fifth aspects of the invention, the genetically modified cell is further modified to constitutively express co-stimulatory receptors.

[0151] As described above, a cellular immune response is typically only induced when an activation signal (typically in response to an antigen) and a co-stimulation signal are simultaneously experienced. Therefore, by having a genetically modified cell in accordance with some of the above embodiments, which includes two or more CARs that in combination provide both an intracellular activation signal and an intracellular co-stimulation signal, ensures that a sufficient immune response can be induce in response to the recognition by the CAR(s) of their cognate antigen. Alternatively, the genetically modified cell may include only one CAR, which has an antigen-recognition domain that recognises a dysfunctional $P2X_7$ receptor, and may constitutively express co-stimulatory receptors, thereby increasing the likelihood of co-stimulation being provided simultaneously when the CAR is activated. Alternatively, the genetically modified cell may be further modified to constitutively express both co-stimulatory receptor(s) and its/their ligand(s). In this way the cell is continuously experiencing co-stimulation and only needs the activation of a CAR, with a signalling domain including a portion from an activation receptor, for immune activation of the cell.

[0152] Therefore in some embodiments of the fourth or fifth aspects of the invention, the genetically modified cell is further modified so as to constitutively express co-stimulatory receptors. In further embodiments, the genetically modified cell is further modified so as to

express ligands for the co-stimulatory receptors, thereby facilitating auto-stimulation of the cell. Examples of CAR-expressing T cells that also express both co-stimulatory receptors and their cognate ligands (so as to induce auto-stimulation) are known in the art and include, *inter alia*, those disclosed in Stephen MT. *et al*. Nat Med, 2007; 13: 1440-9.

[0153] The potency of a genetically modified cell including a CAR can be enhanced by further modifying the cell so as to secrete cytokines, preferably proinflammatory or proproliferative cytokines. This secretion of cytokines provide both autocrine support for the cell expressing the CAR, and alters the local environment surrounding the CAR-expressing cell such that other cells of the immune system are recruited and activated. Consequently, in some embodiments of the fourth or fifth aspects of the invention the genetically modified cell is further modified to secret cytokines. This secretion may be constitutive, or may be inducible upon recognition of a CAR of its cognate antigen of ligand.

[0154] Whilst any one or more cytokines can be selected depending on the desired immune response, preferable cytokines include IL-2, IL-7, IL-12, IL-15, IL-17 and IL-21, or a combination thereof.

[0155] The genetically modified cell of the fourth or fifth aspects of the invention can be any suitable immune cell, or can be a homogeneous or a heterogeneous cell population. In some embodiments, the cell is a leukocyte, a Peripheral Blood Mononuclear Cell (PBMC), a lymphocyte, a T cell, a CD4+ T cell, a CD8+ T cell, a natural killer cell or a natural killer T cell.

[0156] In a sixth aspect, the present invention provides a method of killing a cell expressing a dysfunctional $P2X_7$ receptor, the method including exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified cell having a chimeric antigen receptor, wherein the chimeric antigen receptor is directed against the dysfunctional $P2X_7$ receptor.

[0157] Therefore, in some embodiments of the sixth aspect of the invention, the CAR directly recognises the dysfunctional $P2X_7$ receptor. In other embodiments, the CAR indirectly recognises the dysfunctional $P2X_7$ receptor.

[0158] As used herein the term “directly recognises” includes direct binding of the antigen-recognition domain of the CAR to the dysfunctional $P2X_7$ receptor, or an epitope thereof, when the receptor is present in its natural form. In another non-limiting example, the antigen-recognition domain may directly bind to a processed form of the dysfunctional $P2X_7$ receptor,

which may be presented by antigen presenting molecules such as the major histocompatibility complex (MHC).

[0159] As an alternative to the CAR directly recognising a cell having a dysfunctional $P2X_7$ receptor, the CAR may be directed against a cell having a dysfunctional $P2X_7$ receptor by an indirect means.

[0160] Consequently, in some embodiments of the sixth aspect of the invention, the chimeric antigen receptor recognises the dysfunctional $P2X_7$ receptor via an intermediate. An intermediate may be a molecule such as a probe that binds or interacts directly with the dysfunctional $P2X_7$ receptor. Non-limiting examples of such probes include antibodies, a Fab of an antibody, a scFv, a soluble engineered TCR or an aptamer. The CAR may be able to directly recognise the probe or the probe may have a tag that is recognised by the CAR. In either regard the probe provides the specificity for the target cell (namely a cell having a dysfunctional $P2X_7$ receptor) whilst the genetically modified cell having the CAR provides the efficacy and directs an immune response against the target cell. Alternatively, the intermediate could be a cell endogenous marker which is associated with, or its expression is correlated to, the dysfunctional $P2X_7$ receptor. The dysregulation of the marker may be a cause of or a result of the dysfunction of the $P2X_7$ receptor.

[0161] In some embodiments of the sixth aspect of the invention, the method of killing a cell having a dysfunctional $P2X_7$ receptor further includes the step of exposing the cell having a dysfunctional $P2X_7$ receptor to an intermediate.

[0162] In some embodiments of the sixth aspect of the invention, the intermediate is a probe that binds to a dysfunctional $P2X_7$ receptor and the chimeric antigen receptor recognises the probe. Preferably the probe is an antibody or an aptamer.

[0163] The term “aptamer” as used throughout the specification refers to any oligonucleic acid, polynucleic acid, peptide or polypeptide which specifically binds to, or preferentially forms a complex with, a target (specifically a dysfunctional $P2X_7$ receptor).

[0164] In some embodiments of the sixth aspect of the invention, the probe includes a tag and the chimeric antigen receptor recognises the tag. Examples of a CAR that recognise cells by way of an intermediate are known in the art, for example European patent application EP 2651442.

[0165] In some embodiments of the sixth aspect of the invention, the cell having a dysfunctional $P2X_7$ receptor is within the body of a subject. In some embodiments, the subject is a human. In some embodiments, the method further includes exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified together with an exogenous cytokine.

[0166] In some embodiments of the sixth aspect of the invention, the genetically modified cell is a genetically modified cell autologous to the cell expressing a dysfunctional $P2X_7$ receptorfrom the subject.

[0167] In some embodiments of the sixth aspect of the invention, the cell expressing a dysfunctional $P2X_7$ receptor is within the body of a subject. In some embodiments of the sixth aspect of the invention, the cell expressing a dysfunctional $P2X_7$ receptor is a cancer cell.

[0168] In some embodiments of the sixth aspect, the present invention provides a method of treating or preventing cancer in a subject, the method including providing a subject with a genetically modified cell having a chimeric antigen receptor, wherein the chimeric antigen receptor is directed against a target cell having a dysfunctional $P2X_7$ receptor.

[0169] The terms "treat", "treating" or "treatment," as used herein are to be understood to include within their scope one or more of the following outcomes: (i) inhibiting to some extent the growth of a primary tumour in a subject, including, slowing down and complete growth arrest, and including reducing the growth of the primary tumour after resection; (ii) inhibiting to some extent the growth and formation of one or more secondary tumours in a subject; (iii) reducing the number of tumour cells in a subject; (iv) reducing the size of a tumour in the subject; (v) inhibiting (i.e. reduction, slowing down or complete stopping) of tumour cell infiltration into peripheral organs; (vi) inhibiting (i.e. reduction, slowing down or complete stopping) of metastasis; (vii) improving the life expectancy of a subject as compared to the untreated state; (viii) improving the quality of life of a subject as compared to the untreated state; (ix) alleviating, abating or ameliorating at least one symptom of cancer in a subject; (x) causing regression or remission of cancer in a subject; (xi) relieving a condition in a subject that is caused by cancer; and (xii) stopping symptoms in a subject that are associated with cancer.

[0170] The terms "prevent" or "preventing" as used herein are to be understood to include within their scope inhibiting the formation of a primary tumour in a subject, inhibiting the

formation of one or more secondary tumours in a subject, or reducing or eliminating the recurrence of cancer in a subject in remission.

[0171] The term "inhibiting" as used herein is taken to mean a decrease or reduction in the growth of a cancer, cancerous cell or tumour when compared to the growth in a control, such as an untreated cell or subject. In some embodiments, growth may be decreased or reduced by at least 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100%, relative to an untreated control.

[0172] Inhibition of the growth of a cancer, tumour or cancerous cell may be assessed by a range of methods known in the art. For example, for a cancerous cell *in vitro*, the growth of the cell may be determined by a suitable proliferation assay, or by method which assess the extent of incorporation of tritiated thymidine into cellular DNA over a given period of time. For a tumour or cancerous cell present *in vivo*, the growth of the tumour or cell may be determined for example by a suitable imaging method known in the art.

[0173] The term "subject" as used herein refers to any animal capable of suffering from cancer. Particular subjects of interest are human beings, and scientifically relevant species such as mice, rats, ferrets, guinea pigs, hamsters, non-human primates, dogs, pigs and sheep, or economically relevant animals such as horses, dogs, cats and cattle. In a preferred embodiment of the sixth aspect of the invention, the subject is a human.

[0174] A reference to "providing a subject with" relates to administering to the subject the genetically modified cell. Alternatively, the genetically modified cell may be generated within the subject. For example, the genetically modified cell may be generated *in vivo* such that the subject has an endogenous population of genetically modified cells. Suitable means for such *in vivo* generation are known in the art and include gene therapy of a subject.

[0175] As used throughout the specification a reference to a CAR being "directed" against a target cell having a dysfunctional $P2X_7$ receptor contemplates the selective targeting of an immune response toward a cell based on the cell having a dysfunctional $P2X_7$ receptor. Importantly, such targeting is not limited to direct recognition of the dysfunctional $P2X_7$ receptor by a CAR. That is to say that the CAR itself does not have to directly recognise or bind to the dysfunctional $P2X_7$ receptor but merely has to be able to selectively recognise and be activated by a cell that expresses a dysfunctional $P2X_7$ receptor.

[0176] Therefore, in some embodiments of the sixth aspect of the invention, the CAR directly recognises the dysfunctional $P2X_7$ receptor. In other embodiments, the CAR indirectly recognises the dysfunctional $P2X_7$ receptor.

[0177] As used herein the term "directly recognises" includes direct binding of the antigen-recognition domain of the CAR to the dysfunctional $P2X_7$ receptor, or an epitope thereof, when the receptor is present in its natural form. In another non-limiting example, the antigen-recognition domain may directly bind to a processed form of the dysfunctional $P2X_7$ receptor, which may be presented by antigen presenting molecules such as the major histocompatibility complex (MHC).

[0178] As an alternative to the CAR directly recognising a cell having a dysfunctional $P2X_7$ receptor, the CAR may be directed against a target cell having a dysfunctional $P2X_7$ receptor by an indirect means.

[0179] Consequently, in some embodiments of the sixth aspect of the invention, the chimeric antigen receptor recognises the dysfunctional $P2X_7$ receptor via an intermediate. An intermediate may be a molecule such as a probe that binds or interacts directly with the dysfunctional $P2X_7$ receptor. Non-limiting examples of such probes include antibodies, a Fab of an antibody, a scFv, a soluble engineered TCR or an aptamer. The CAR may be able to directly recognise the probe or the probe may have a tag that is recognised by the CAR. In either regard the probe provides the specificity for the target cell (namely a cell having a dysfunctional $P2X_7$ receptor) whilst the genetically modified cell having the CAR provides the efficacy and directs an immune response against the target cell. Alternatively, the intermediate could be a cell endogenous marker which is associated with, or its expression is correlated to, the dysfunctional $P2X_7$ receptor. The dysregulation of the marker may be a cause of or a result of the dysfunction of the $P2X_7$ receptor.

[0180] In some embodiments of the sixth aspect of the invention, the method of treating or preventing cancer in a subject further includes the step of providing the subject with an intermediate.

[0181] In some embodiments of the sixth aspect of the invention, the intermediate is a probe that binds to a dysfunctional $P2X_7$ receptor and the chimeric antigen receptor recognises the probe. Preferably the probe is an antibody or an aptamer.

[0182] The term "aptamer" as used throughout the specification refers to any oligonucleic acid, polynucleic acid, peptide or polypeptide which specifically binds to, or preferentially forms a complex with, a target (specifically a dysfunctional $P2X_7$ receptor).

[0183] In some embodiments of the sixth aspect of the invention, the probe includes a tag and the chimeric antigen receptor recognises the tag. Examples of a CAR that recognise cells by way of an intermediate are known in the art, for example European patent application EP 2651442.

[0184] In a seventh aspect, the present invention provides a method of treating or preventing cancer in a subject, the method including administering to the subject a genetically modified cell according to a fourth or fifth aspect of the invention.

[0185] Whilst the provision of a genetically modified cell expressing a CAR directed against a target cell having a dysfunctional $P2X_7$ receptor may be sufficient to provide effective immunotherapy against precancerous or cancerous cells, the provision of adjuvants together with the genetically modified cells may further enhance the induction of the immune response and may augment the immunotherapy. Cytokines, preferably proinflammatory cytokines, are particularly suitable adjuvants for provision to a subject together with genetically modified cells having CARs.

[0186] Therefore, in some embodiments of the sixth and seventh aspects of the invention, the genetically modified cell is administered to the subject together with a cytokine. It is to be understood that as used throughout the specification the term "together with" includes the genetically modified cell being administered simultaneously with a cytokine or administered in combination with a cytokine. Consequently, when administered in combination with a cytokine this may be considered to include a combination therapy whereby a subject's immunotherapy includes both treatment with a cytokine and treatment with a genetically modified cell having a CAR directed against a target cell expressing a dysfunctional $P2X_7$ receptor. In some forms, the cytokine is administered on a different day (>24hrs) to the administration of the genetically modified cells. In other forms the cytokine is administered on the same day (within 24hrs) as the genetically modified cells. In further forms the cytokine(s) and the genetically modified cell is administered within 18hrs, 12hrs, 6hrs, 4hrs, 2hrs, 1hr, 45mins, 30mins, 15mins, 10mins, 5mins, 2mins or 1min of each other.

[0187] Suitable cytokines for administration together with the genetically modified cell include IL-2, IL-4, IL-6, IL-7, IL-9, IL-12, IL-15, IL-17, IL-18, IL-21, IL-23, IFNα, IFNβ, IFNγ, GM-CSF,

TGFβ and TNFα. Preferred cytokines include IL-2 and IFNα. Furthermore, the cytokines may be administered as recombinant forms, natural forms, or via delivery systems such as fusions with proteins, delivered as a nucleic acid sequence which is expressed in the genetically modified cell or conjugated with a polymer such a polyethylene glycol (PEG).

[0188] The cell to be genetically modified can be obtained from any suitable source. In some embodiments of the sixth or seventh aspects of the invention the cell to be genetically modified is an autologous cell, being a cell autologous to the cell expressing a dysfunctional $P2X_7$ receptor. Advantageously, an autologous cell would not be recognised as 'non-self' by the subject's immune system and would therefore be tolerated by the subject. However, in some forms of cancer suitable autologous cells may not be readily available. Therefore, in some embodiments of the invention the cell to be genetically modified is an allogeneic or heterologous cell.

[0189] $P2X_7$ dysfunction is a common molecular alteration in a variety of cancers. Consequently, the method of the sixth or seventh aspects of the invention can be used for the prevention and treatment of a variety of cancers.

[0190] In some embodiments of the sixth or seventh aspects of the invention the method is used for the prevention or treatment of a cancer selected from one or more of; brain cancer, oesophageal cancer, mouth cancer, tongue cancer, thyroid cancer, lung cancer, stomach cancer, pancreatic cancer, kidney cancer, colon cancer, rectal cancer, prostate cancer, bladder cancer, cervical cancer, epithelial cell cancers, skin cancer, leukaemia, lymphoma, myeloma, breast cancer, ovarian cancer, endometrial cancer, testicular cancer. Preferably the cancer is selected from one or more of lung cancer, oesophageal cancer, stomach cancer, colon cancer, prostate cancer, bladder cancer, cervical cancer, vaginal cancers, epithelial cell cancers, skin cancer, blood-related cancers, breast cancer, endometrial cancer, uterine cancer testicular cancer..

[0191] In some embodiments of the sixth or seventh aspects of the invention, the cancer is metastatic cancer, such as stage III or stage IV cancer.

[0192] Upon creation of a genetically modified cell in accordance with the fourth or fifth aspects of the invention it may be desirable to expand the cell population *in vitro* to increase the total cell numbers available for use in treatment. This can be done using the step of exposing the cell to an antigen for the CAR. Accordingly, in an eighth aspect the present invention provides a method of expanding *in vitro* the genetically modified cell according to

the fourth or fifth aspects of the invention, the method including the step of exposing the cell to an antigen for the CAR. In some embodiments, the method includes the further step of exposing the cell to a cytokine.

[0193] In a ninth aspect, the present invention provides a method of expanding *in vitro* the genetically modified cell according to the fourth or fifth aspects of the invention, the method including the step of exposing the cell to an antigen for the CAR and simultaneously exposing the cell to a cytokine.

[0194] Preferable cytokines used in the eighth or ninth aspects of the invention include members of the IL-2 subfamily, the interferon subfamily, the IL-10 subfamily, the IL-1 subfamily, the IL-17 subfamily or the TGF-β subfamily. In some embodiments of the eighth or ninth aspects of the invention, the cytokine is selected from the group consisting of IFN-γ, IL-2, IL-5, IL- 7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, TNF-α, TGF-β1, TGF-β2, TGF-β3 and GM-CSF, or a combination thereof.

[0195] In a tenth aspect, the present invention provides a method of expanding *in vitro* the genetically modified cell according to the fourth or fifth aspects of the invention, the method including exposing the cell to immobilised anti-CD3 and anti-CD28 antibodies. In some embodiments of the tenth aspect of the invention, the antibodies are immobilised on a beaded substrate (for example “Human Activator” Dynabeads™). In some embodiments of the tenth aspect of the invention, the antibodies are immobilised on an alternative surface such as the surface of a tissue culture vessel, a culture flask, plate or bioreactor.

[0196] As would be understood by a person skilled in the art, depending on the signalling domain of the CAR, recognition by the CAR of its cognate antigen will lead to intracellular signalling that may ultimately lead to cellular proliferation. Accordingly, small numbers of cells, or even individual cells, can be expanded (or in the case of a single cell, clonally expanded) to form therapeutically significant numbers. This process can be further enhanced by the provision of cytokines.

[0197] The delivery or administration of the genetically modified cell according to a fourth or fifth aspect of the invention may be delivery or administration of the cell alone, or delivery or administration of the cell formulated into a suitable pharmaceutical composition. Accordingly, in an eleventh aspect, the present invention provides a pharmaceutical composition including a genetically modified cell according to a fourth or fifth aspect of the invention, and a pharmaceutically acceptable carrier.

[0198] Methods are known in the art for providing CAR-containing cells for immunotherapy (see for example Kershaw, MH. *et al*. Clin Cancer Res. 2006;12(20): 6106-15; Parker LL. *et al*. Hum Gene Ther 2000;11: 2337-87). Furthermore, protocols and methods are known in the art for the preparation, expansion and assessment of mammalian CAR-expressing cells (see for example Cheadle, EJ. *et al*. Antibody Engineering: Methods and Protocols, Second Edition, Methods in Molecular Biology, vol. 907: 645-66) and are summarised in the Examples below.

[0199] The pharmaceutical composition may also include one or more pharmaceutically acceptable additives, including pharmaceutically acceptable salts, amino acids, polypeptides, polymers, solvents, buffers, excipients and bulking agents, taking into consideration the particular physical and chemical characteristics of the cell to be administered. In some embodiments, the pharmaceutical composition includes a suspension of genetically modified cells according to the fourth or fifth aspects of the invention in a suitable medium, such as isotonic saline solution. In some embodiments, the pharmaceutical composition may include suitable adjuvants such as one or more cytokines as described above. In some embodiments, the pharmaceutical composition may also include an intermediate as described above.

[0200] Administration of the pharmaceutical composition may also be via parenteral means which include intravenous, intraventricular, intraperitoneal, intramuscular or intracranial injection, or local injections to the site of a tumour or cancerous mass.

[0201] Throughout this specification, unless the context requires otherwise, the word “comprise”, or variations such as “comprises” or “comprising”, will be understood to imply the inclusion of a stated element or integer or group of elements or integers but not the exclusion of any other element or integer or group of elements or integers.

[0202] Finally, reference is made to standard textbooks of molecular biology that contain methods for carrying out basic techniques encompassed by the present invention. See, for example, Green MR and Sambrook J, *Molecular Cloning: A Laboratory Manual* (4th edition), Cold Spring Harbor Laboratory Press, 2012.

[0203] It will be apparent to the person skilled in the art that while the invention has been described in some detail for the purposes of clarity and understanding, various modifications and alterations to the embodiments and methods described herein may be made without departing from the scope of the inventive concept disclosed in this specification.

[0204] The invention is further illustrated in the following examples. The examples are for the purpose of describing particular embodiments only and are not intended to be limiting with respect to the above description

EXAMPLE 1

Protocol for PEP2-2-3 binding peptide Chimeric Antigen Receptor (CAR) Design and Expression

[0205] An exemplified protocol detailing the process of designing and expressing an anti-non-functional (nf) $P2X_7$ receptor CAR according to an embodiment of the present invention is detailed as follows.

Design of PEP2-2-3 (anti-nf $P2x_7$) Chimeric Antigen Receptor

[0206] An anti-nf$P2x_7$ chimeric antigen receptor (CAR) was designed according to the schematic illustrated in Figure 1.

[0207] An antigen-recognition domain **1** of the CAR was generated which included the amino acid sequence of the PEP2-2-3 binding peptide (amino acid sequence set forth in SEQ ID NO: 10 and nucleotide sequence set forth in SEQ ID NO: 11). The PEP2-2-3 sequence was shown to have specific affinity for the dysfunctional $P2X_7$ receptor expressed on cancer cells, such as prostate LNCap cells, without significant affinity for monocytes or lymphocytes.

[0208] A CD8a signalling peptide **2** (having the amino acid sequence set forth in SEQ ID NO: 12 and the nucleotide sequence set forth in SEQ ID NO: 13) was linked to the N-terminus of the PEP2-2-3 antigen-recognition domain **1**. The CD8a signalling peptide **2** includes a Kozak consensus sequence at positions 1 to 13 of SEQ ID NO: 13. The CD8a signalling peptide **2**, including the Kozak sequence, acts to facilitate recognition of the transcribed RNA by the ribosome and provides a translation start site, thereby promoting translation of the transcribed RNA sequence of the CAR to a protein.

[0209] The antigen-recognition domain **1** of the CAR was linked to a transmembrane domain **3** via one of two hinge regions, termed long hinge **4** and short hinge **5**. The provision of a long hinge **4** permits for flexibility of the antigen-recognition domain which may be required for the antigen-recognition domain to interact with its cognate ligand (dysfunctional $P2X_7$). The amino acid and nucleotide sequences for the long hinge **4** are set forth in SEQ ID NO: 14 and SEQ ID NO: 15, respectively. The amino acid and nucleotide sequences for the short hinge **5** are set forth in SEQ ID NO: 16 and SEQ ID NO: 17, respectively.

[0210] The transmembrane domain **3**, and a portion of the intra-cellular domain **6**, of the CAR is provided by a portion of the CD28 co-stimulatory receptor **7** (amino acid sequence set forth in SEQ ID NO: 18 and nucleotide sequence set forth in SEQ ID NO: 19). The intracellular domain further includes a portion of the co-stimulatory receptor OX40 **8** (amino acid sequence set forth in SEQ ID NO: 20 and nucleotide sequence set forth in SEQ ID NO: 21) and a portion of the activation receptor CD3 zeta **9** (amino acid sequence set forth in SEQ ID NO: 22 and nucleotide sequence set forth in SEQ ID NO: 23).

[0211] A P2A sequence **10** (amino acid sequence set forth in SEQ ID NO: 24 and nucleotide sequence set forth in SEQ ID NO: 25) was added to the C-terminus of the CAR, permitting the post-translational excision of any peptide sequence attached to the C-terminus of the CAR. The amino acid sequence for the constructed anti-nf$P2X_7$ CAR-long hinge and the anti-nf$P2X_7$ CAR-short hinge are set forth in SEQ ID NOs: 26 and 27, respectively.

Lentival Vector Design and Assembly

[0212] The designed CARs were incorporated into the BLIV lentiviral plasmid (System Biosciences, California, USA) illustrated in Figure 2, which includes the fluorescence and bioluminescence reporting proteins, green-fluorescence protein (GFP) and Firefly Luciferase (FLuc). The BLIV plasmid further includes a T2A coding sequence between the GFP and FLuc reporter protein coding sequences permitting for post-translational separation of the FLuc and GFP proteins.

[0213] Sequences having homology to the sequences upstream and downstream of the *Nhe*I restriction site of the BLIV vector were added to the 5' and 3' ends of the designed CARs to result in the final nucleotide sequences set forth in SEQ ID NO 28 (CAR-long hinge) and SEQ ID NO: 29 (CAR-short hinge). The inclusion of the 5' and 3' sequences permitted incorporation of the anti-nf $P2X_7$ CAR into the BLIV vector using Gibson cloning.

[0214] The nucleotide sequence for the anti-nf $P2X_7$ CAR-long hinge and anti-nf $P2X_7$ CAR-short hinge were constructed using gene blocks technology (gBlock™ Gene Fragments - Integrated DNA Technologies, Iowa, USA) and assembled using Gibson Assembly Cloning Kit (New England Biolabs inc. Ipswich MA, USA – cat# E5510S) in accordance with the manufacturer's instructions.

[0215] The BLIV plasmid was restricted at the *Nhe*I cloning site and the anti-nf $P2X_7$ CAR coding sequence was incorporated using Gibson assembly.

Cloning and evaluation of BLIV-CAR vector

[0216] New England Biolabs 5-alpha Competent *E. coli* cells (provided in Gibson Assembly Cloning Kit) were transformed with the generated BLIV-CAR vectors as per the manufacturer's instructions. Briefly:

- A tube of NEB 5-alpha Competent E. coli cells was thawed on ice for 10 minutes;
- 1-5 µl containing 1 pg – 100 ng of BLIV-CAR plasmid DNA was added to the cell mixture and mixed in by flicking the tube 4 to 5 times;
- The *E.coli* and plasmid mixture was placed on ice for 30 minutes without mixing;
- The cell and plasmid mixture were heat shocked at 42°C for 30 seconds before being placed on ice for 5 minutes without mixing;
- 950 µl of SOC was added to the mixture before being heated to 37°C for 60 minutes and shaken vigorously;
- Selection plates were prepared and heated to 37°C;
- Serial 10-fold dilutions of the cells were prepared in SOC solution; and
- 50 to 100 µl of each dilution was spread onto selection plates followed by incubation overnight at 37°C.

[0217] Following incubation of the transformed (*E.coli*) cells, 10 colonies of bacteria transformed with BLIV-CAR-short hinge plasmid and 10 colonies of bacteria transformed with the BLIV-CAR-Long hinge plasmid were isolated, plasmid DNA was purified, and restricted with a *Bam*HI restriction enzyme. The restricted DNA was analysed via gel electrophoresis for appropriate sized restriction fragments. As shown in Figure 3, colonies 2 to 9 of the bacterial clones transformed with the BLIV-CAR-long hinge plasmid contained the appropriately sized restriction fragments (7.8kb and 2.8kb), while only colony 4 of the bacterial clones transformed with the BLIV-CAR-short hinge plasmid provided the appropriate sized restriction fragments (7.4kb and 2.8kb).

[0218] Clones 2 to 4 of the bacteria containing the BLIV-CAR-long hinge plasmid (L2 to L4), and clone 4 of the bacteria containing the BLIV-CAR-short hinge plasmid (S4) were selected for further confirmation of plasmid identity using *Eco*RI, *Bam*HI and *Pst*I restriction enzymes. All of the colonies showed restriction fragments of the expected length, as set out in Table 4 and Figure 4.

TABLE 4

Expected Restriction Fragment Length of BLIV-CAR Plasmids

Restriction Enzyme and Plasmid	Expected Length
Unmodified BLIV plasmid	8.9kb
Bam HI restricted BLIV-CAR-long hinge	7.8kb and 2.8kb
Bam HI restricted BLIV-CAR-short hinge	7.4kb and 2.8kb
Eco RI restricted BLIV-CAR-long hinge	6.8kb, 2.6kb and 1.5kb
Eco RI restricted BLIV-CAR-short hinge	7.7kb and 2.6kb
Pst I restricted BLIV-CAR-long hinge	8.6kb, 2.0kb and 0.22kb
Pst I restricted BLIV-CAR-short hinge	9.3kb, 0.8kb and 0.22kb

Construction and Verification of Lentiviral Vectors

[0219] 293T cells were used to package lentivirus from a 3 plasmid protocol according to the following method.

Day 1: 293T cells were seeded in 35ml DMEM media with 10% serum in a T-225 flask such that the cells were 90-95% confluent the following day.

Day 2: 30ug of one of the generated BLIV-CAR plasmids (or an unmodified BLIV plasmid), 30ug of gag-pol plasmid delta 8.2, and 15ug of VSV-G plasmid (pMD2.G), were added to OptiMEM media to a final volume of 750ul, and mixed. 300ul of PEI solution were added and incubated at room temperature for at least 20 minutes. The mixture was then added to the confluent 293T cells before incubation at 37°C.

Day 3: Supernatant was decanted from the 293T cells 24 hours after addition of the plasmid mixture and stored at 4°C. The decanted mixture was replaced with 35ml of fresh media before further incubation at 37°C.

Day 4: 48 hours after addition of the plasmid mixture, the media was removed and combined with the supernatant from the 24 hour harvest. The combined supernatants were spun for 15 minutes at 1500g to remove any remaining cellular debris. The supernatant was filtered through a 0.45um filter, and then spun at 17,000rpm in a WX ultracentrifuge for one hour. After centrifugation, the supernatant was decanted by hand, with 50-200ul remaining in the tube. The centrifuge tube was placed in a 50ml screw-top tube in order to prevent contamination and evaporation and the virus was allowed to resuspend at 4°C overnight.

Day 5: The virus was resuspended off the bottom of the centrifuge tube and transferred into a new 1.5ml tube. The resuspended virus was spun for 5 minutes in a microcentrifuge tube at 5000 rpm to remove any remaining debris.

[0220] Transfection of 293T cells with the BLIV-CAR-short hinge and BLIV-CAR- long hinge vector was assessed after 24 hours of incubation by the presence of GFP fluorescence (see Figure 5A and Figure 6A). Supernatant collected at day 5 (as set out above) containing short- and long-hinge BLIV-CAR lentivirus vectors were incubated with fresh 293T cells and visualized for GFP fluorescence to test transduction capacity (see Figure 5B and Figure 6B).

Screening for CAR T cell function

[0221] 10^8 CD8 T cells were isolated from 50ml of human blood using the RosetteSep™ human CD8+ T cell isolation kit (Stemcell technologies, Vancouver, Canada) in accordance with the manufacturer's instructions. Analysis of the purity, as illustrated in Figure 7, demonstrated that 76.6% of purified cells were CD8+

[0222] CD8+ T cells were incubated at 10^5 cells per well with a 1:1 ratio of dynal T cell expander (CD3/CD28) beads. The CD8 cells were then incubated overnight together with lentiviral preparations, at a multiplicity of infection (MOI) of 5 or greater, containing either unmodified BLIV plasmids, BLIV-CAR–short hinge plasmids or BLIV-CAR-long hinge plasmids. Following incubation, the CD8+ T cells were washed before being co-cultured with the target cells.

[0223] Target cells expressing the non-functional $P2X_7$ receptor were provided by the mammary cancer cell line BT549 (ATCC HTB-122). These cells were dye-labelled using the fluorescent membrane intercalculating dye eFluor™ 670 (affymetrix eBioscience) as per the manufacturer's instructions. Briefly:

- BT549 cells were prepared as a single-cell suspension and washed in PBS twice to remove any residual serum;
- Cells were resuspended in room temperature PBS;
- A 10 μM solution of Cell Proliferation Dye eFluor® 670 was prepared in room temperature PBS;
- An equal volume of the 10 μM dye solution was added to the prepared BT549 cells to give a final concentration of 5 μM dye solution;
- The BT549 cells in the dye solution were incubated for 10 minutes at 37°C in the dark, before the labelling was stopped by adding 4 times the volume of cold culture medium containg 10% serum followed by incubation on ice for 5 minutes in the dark;

- Finally cells were washed 3 times in culture medium before being resuspended in culture medium at the desired concentration.

[0224] Following dye labelling, target cells were co-culturing with the prepared CD8+ T cells at ratios of 10:1, 5:1, 1:1 and 0:1 (T cells:targets).

[0225] After 24hrs of co-culture, cells were collected and analysed using Fluorescence-Activated Cell Sorting (FACS). The number of target cells containing the membrane intercalculating dye was quantified to assess if the co-cultured T cells led to target cell death or arrest of cell proliferation. The gating and analysis strategy used to quantify the efficacy of the CD8+ T cells at killing target cells is illustrated in Figure 8, and is quantified in Figure 9. Figure 8A illustrates the gating and histogram analysis of labelled CD8+ T cells. Figure 8B illustrates the gating and histogram analysis of labelled BT549 target cells. Figure 8C illustrates the gating and histogram analysis after 24hrs of co-culture of control CD8+ T cells and BT549 targets. Figure 8D illustrates the gating and histogram analysis after 24hrs of co-culture of CD8+ T cells transduced with BLIV-CAR-long hinge and BT549 target cells. Figure 8E illustrates the gating and histogram analysis of 24hr co-culture of CD8+ T cells transduced with BLIC-CAR-short hinge and BT549 target cells.

[0226] As can be seen in Figure 9, there was an increase in the number of BT549 target cells deleted (killed) when the target cells were co-cultured with CD8 T cells transduced with lentivirus containing either the BLIV-CAR-long hinge or BLIV-CAR-short hinge, compared to the co-culture of the target cells with non-transduced or control transduced (unmodified BLIV vector) CD8 T cells.

[0227] In view of the results presented in Figure 9, it is apparent that CD8+ T cells transduced with anti-nfP2X$_7$ CAR receptors (having either the short or the long hinge) demonstrate elevated levels of cytotoxic activity towards non-functional P2X$_7$ expressing target cells, demonstrating the ability of the CAR-T cells to kill cancer cell targets.

EXAMPLE 2

Design of alternative anti-nfP2X7 Chimeric Antigen Receptor

[0228] A further exemplified protocol detailing the process of designing, and expressing on a T cell, an anti-non-functional (nf) P2X$_7$ receptor CAR, according to an embodiment of the present invention, is detailed below.

[0229] Anti-nfP2X$_7$ CARs were designed utilising three anti-non-functioning P2X$_7$ binding peptides. Specifically, CARs were designed to include antigen recognition domains with sequence homology to peptides PEP2-2-1-1, PEP2-472-2 or PEP2-2-12 (having the amino acid sequences set forth in SEQ ID NOs: 32, 33 and 34 respectively). These binding peptides have been shown to bind to the non-functional P2X7 receptor (Barden, J.A., Sluyter, R., Gu, B.J. & Wiley, J.S. 2003. Specific detection of non-functional human P2X(7) receptors in HEK293 cells and B-lymphocytes. FEBS Lett 538, 159-162).

[0230] The alignment of the above binding peptides to the heavy chain variable regions of antibodies that recognise non-functional P2X$_7$ receptors is shown in Figure 10. The alignment of the Complementarity Determining Region (CDR 1 to 3) sequences are indicated by the boxes.

[0231] A specific example of the construction of a CAR having the PEP2-2-1-1 sequence is detailed below. The same CAR structure and sequences were used for CARs having PEP2-472-2 or PEP2-2-12 sequences as the binding peptides, with the alternative binding peptides substituted for PEP2-2-1-1.

[0232] DNA sequences coding for the PEP2-2-1-1 binding peptide were synthesised in-frame with other DNA sequences to generate a CAR having the configuration described below.

[0233] With reference to Figure 11, an antigen recognition domain was prepared by linking a leader sequence of Homo sapiens CD8a molecule (CD8A) transcript variant 1 (having the amino acid sequence set forth in SEQ ID NO: 30 and the nucleotide sequence set forth in SEQ ID NO: 31) **11** to the N terminus of the PEP2-2-1-1 binding peptide **12** (having the amino acid sequence set forth in SEQ ID NO: 32 and the nucleotide sequence set forth in SEQ ID NO: 35).

[0234] The antigen recognition domain was then linked to a transmembrane domain via a modified IgG4 Hinge-CH2-CH4 **13** having the sequence of the long hinge as set out in Example 1 above (i.e. the amino acid sequence set forth in SEQ ID NO: 14 and the nucleotide sequence set forth in SEQ ID NO: 15).

[0235] The extracellular domain comprising the CD8 leader sequence **11** and the PEP2-2-1 binding peptide **12** was linked to a transmembrane domain **14** provided by a portion of human CD28 **15** (having the amino acid sequence set forth in SEQ ID NO: 18 and the nucleotide

sequence set forth in SEQ ID NO: 19) which also included a portion of the CD28 cytoplasmic domains **16**.

[0236] The intracellular portion of the CAR **17** was provided by a portion of the human CD28 molecule mentioned above **14** and the cytoplasmic domain of Homo sapiens tumour necrosis factor receptor superfamily member 4 (TNFRSF4/OX40 – having the amino acid sequence set forth in SEQ ID NO: 20 and the nucleotide sequence set forth in SEQ ID NO: 21) **18** linked to the cytoplasmic domain of Homo sapiens CD247 molecule **19** (T-cell surface glycoprotein CD3 zeta chain, having the amino acid sequence set forth in SEQ ID NO: 22 and the nucleotide sequence set forth in SEQ ID NO: 23).

Lentival Vector Design and Assembly

[0237] The nucleotide sequences for the designed PEP2-2-1-1, PEP2-472-2 and PEP2-2-12 CARs were constructed using gene blocks technology (gBlock™ Gene Fragments - Integrated DNA Technologies, Iowa, USA) and assembled using Gibson Assembly Cloning Kit (New England Biolabs inc. Ipswich MA, USA – cat# E5510S) in accordance with the manufacturer's instructions. The sequences of the nucleotide constructs for PEP2-2-1-1, PEP2-472-2 or PEP2-2-12 CARs for integration in to cloning vectors (including restriction sites) are set out in SEQ ID NOs: 35, 36 and 37, respectively.

[0238] The CAR nucleotide constructs were incorporated into the pCDH-CMV-MCS-T2A (pCDH) vector (System Biosciences, California, USA Cat# CD524A-1) illustrated in Figure 11, which includes the fluorescence reporting protein, green-fluorescence protein (GFP). The pCDH vector further includes a T2A coding sequence between the cloning site and the GFP permitting for post-translational separation of the cloned CAR and the GFP proteins.

[0239] For integration of PEP2-2-12 and PEP2-472-2 CAR nucleotide constructs into the pCDG vector, the pCDH vector was restricted with *EcoR1* and *NotI* and gel purified (QIAquick gel extraction kit, QIAGEN). PEP2-2-12 and PEP2-472-2 CAR nucleotide gBlock constructs were also digested with *EcoRI* and *NotI* digestion enzymes. The restricted gBlock fragment was then purified with a QIAquick PCR purification kit in accordance with the manufacturer's instructions. The restricted vector was ligated with the restricted CAR constructs at a 3:1 molar ratio of insert to vector. Ligation mixes were transformed into chemical competent SURE2 cells (Agilent).

[0240] The PEP2-2-1-1 CAR construct contained an internal *EcoR1* restriction site and therefore it was integrated into the pCDH vector in a manner different to the PEP2-2-12 and

PEP2-472-2 CAR nucleotide constructs. The pCDH vector was restricted with *EcoR1*, and the resulting 5'-overhang filled by T4 DNA polymerase in the presence of 100uM dNTPs (12°C for 15 minutes). The reaction was terminated (75°C for 20 minutes in the presence of 10mM EDTA) and the restricted vector was column purified (QIAquick PCR purification kit, QIAGEN). The purified vector was then further restricted with *NotI* and gel purified (QIAquick gel extraction kit, QIAGEN). The PEP2-2-1-1 CAR construct fragment was first restricted with *SmaI* followed by *NotI* digestion (both at 25°C). The restricted gBlock fragment was purified with a QIAquick PCR purification kit in accordance with manufacturer's instructions. The restricted vector was ligated with the CAR construct at a 3:1 molar ratio of insert to vector.

Cloning and evaluation of pCDH-CAR vector

[0241] Ligation mixes for each of the three CAR constructs described above were transformed into chemical competent SURE2 cells (Agilent) in accordance with the manufacturer's instructions. Briefly:

- SURE2 cells were thawed on ice. Once thawed, the cells were gently mixed and 100 µl aliquots of cells were placed in pre-chilled 14ml round bottom tubes;
- 2µl of the β-Mercaptoethanol was added to each aliquot of cells;
- The tubes were mixed and incubated on ice for 10 minutes, swirling gently every 2 minutes;
- 0.1-50ng of each of the pCDH-CAR vectors was added to an aliquot of cells;
- The aliquots were gently mixed, then incubate on ice for 30 minutes;
- The tubes were heat-pulsed at 42°C in a water bath for 30 seconds and then incubate on ice for 2 minutes;
- 0.9ml of preheated (42°C) NZY+ broth was added to each tube followed by incubation at 37°C for 1 hour with agitation at 225-250 rpm;
- Up to 200µl of the transformation mixture was plated on LB agar plates containing antibiotic, followed by incubation at 37°C overnight;
- Colonies were picked and cultured further overnight;
- Plasmid DNA was isolated from cultured clones with a Quicklyse miniprep kit (QIAGEN) and digested with *EcoRI/Not I* digestion to identify clones with the correctly sized CAR-pCDH vectors

[0242] Following incubation of the transformed (SURE2) cells, 5 to 6 colonies of cells transformed with pCDH-CAR for each of the PEP2-2-1-1, PEP2-472-2 or PEP2-2-12 binding peptides were isolated and further incubated overnight. Plasmid DNA was isolated with a

Quicklyse miniprep kit (QIAGEN) from each of the cultured colonies, and restricted with the *EcoRI/Not I* restriction enzymes. The restricted DNA was analysed via gel electrophoresis for appropriate sized restriction fragments.

[0243] As shown in Figure 13, colony 3 of the PEP2-2-1-1 pCDH-CAR construct, colonies 1 and 3 of the PEP2-472-2 pCDH-CAR construct and colonies 1, 3 and 5 of the PEP2-2-12 pCDH-CAR construct contained the appropriately sized restriction fragments.

[0244] Each selected clone was sequenced to confirm the integration of the CAR using the appropriate primers selected from Table 5.

TABLE 5

Primers used for confirmation of correct CAR construct in selected colonies

Primer name	Sequence	SEQ ID NO
pCHD-CMV-For	GGTGGGAGGTCTATATAAGC	SEQ ID NO: 38
pCHD-coGFP-Rev	TGATGCGGCACTCGATCTC	SEQ ID NO: 39
2-2-1-1-Rev	CTTCACGGAGTCTGCGTAG	SEQ ID NO: 40
2-2-1-1-For	TCTTGTCACTGTATCCAGTG	SEQ ID NO: 41
2-472-2-Rev	CGTATCTTCAGCTCTCAAGC	SEQ ID NO: 42
2-472-2-For	TGGTCCTTCAGTTTTCCTGT	SEQ ID NO: 43
2-12-2-Rev	CAGCTGTATCTTCTGCTC	SEQ ID NO: 44
Com-For-1	AGTGGGAGAGTAACGGACAG	SEQ ID NO: 45
Com-For-2	AGGGCCAGAATCAATTGTAC	SEQ ID NO: 46

[0245] Sequencing data for each selected colony was aligned to the *in silico* derived recombinant clone for each of the PEP2-2-1-1, PEP2-472-2 or PEP2-2-12 CAR constructs and appropriate constructs were verified for at least one of each of the selected colonies. Large scale Endotoxin free plasmid isolation of the verified clones was performed with a NucleoBond® Xtra Midi EF kit, Macherey-Nagel in accordance with the Manufacturer's instructions.

Construction and Verification of Viral Vectors

[0246] Lentivirus packaging was performed in transiently transfected Hek293T cells using Lipofectamine 2000 reagent (Invitrogen) as per a standard laboratory protocol (Brown, C.Y. *et al.* 2010. Robust, reversible gene knockdown using a single lentiviral short hairpin RNA vector. *Hum Gene Ther* 21, 1005-1017). Briefly:

- 12.5ug of Lentiviral vector DNA was mixed with 3.75ug of pMD2.g (VSV-G envelope expression vector), 6.25ug of pRSV-Rev and 7.5ug of pCMVdelta8.2 per transfection in a T75cm flask using 75ul of Lipofectin as per the manufacturer's protocol, and incubated overnight;
- The following morning media was changed and virus containing supernatant was harvested 48 hours later;
- Harvested supernatants were centrifuged at 300xg for 5 minutes before being filtered through a 0.45um filter;
- Virus particles from filtered supernatants were concentrated by ultracentrifugation (68,000xg for 90 minutes and 4°C, Beckman SW32 rotor). The supernatant was removed and the virus pellet resuspended gently in DMEM on ice;
- 100ul virus aliquots were stored at -70°C until needed.

[0247] To assess the rate of virus transfection, transfected Hek293T cells were harvested and the percent of GFP positive cells (pCDH vector containing cells) was determined by flow cytometry. Representative results for Hek293T transfected with the LV-PEP2-472-2 packaging mix is shown in Figure 14.

[0248] Viral titres were calculated by transduction of a known number of Hek293T cells with serial dilutions (1:50 and 1:100) of concentrated LV stock. Transductions were performed overnight in the presence of 8ug/ml polybrene (Hexadimethrine bromide). The following day media containing virus and polybrene was replaced with fresh media, cells were harvested 24 hours later and the percentage of GFP positive cells determined by flow cytometry. Viral titres were calculated using the formula: Transduction units/ml (TU) = (FxC/V)xD where F= frequency of GFP+ cells (%GFP+/100), C= cell number at the time of virus addition, V= volume of transduction in mL and D= dilution factor. Representative flow data for LV-PEP2-472-2 transduction is shown in Figure 15. The TU for each of the PEP2-2-1-1, PEP2-12-2 and PEP2-472-2 CAR viral vectors is provided in the Table 6 below.

TABLE 6

Transduction Units for Viral Vectors

Lentiviral construct	Dilution Factor	Average %GFP$^+$ cells	TU/ml
PEP2-2-1-1	50	31.15	4.14e6
	100	21.25	5.62e6
PEP2-12-2	50	54.6	7.26e6
	100	33.15	8.82e6

PEP2-472-2	50	62	8.25e6
	100	39.55	10.52e6

Screening for nf-P2X$_7$ CAR T Cell Function

Production of CD8 T Cells Expressing Anti-nf-P2X$_7$ CARs

[0249] Human CD8 cells were purified and transduced in accordance with the following method:

[0250] Human CD8 T cells were purified from mononuclear cells (MNCs) isolated from Buffy Coats from anonymous donors (Australia Red Cross blood service). MNCs were isolated using Ficoll-Paque™ density gradient media. CD8 T cells were purified from MNCs Dynabeads® Untouched™ Human CD8 T Cells Kit (Invitrogen) according manufacturer's instructions. The purity of isolated cells, as assessed by flow cytometry, was ≥85%.

[0251] 2x10^6 purified cells were pre-incubated with CD3/CD28 beads (3:1 bead to cell ratio) and IL2 (500U/ml) for 30 minutes prior to the addition of 1 to 2 multiplicity of infection (MOI) units of virus containing LV-PEP2-2-1-1, LV-PEP2-472-2 or an empty LV vector (GFP control virus) together with 8ug/ml polybrene. Cells were incubated with virus for 16 hours before the virus containing media was removed. The remaining cells and beads were incubated in fresh media including IL2 for 40 hours before GFP fluorescence levels were analysis.

[0252] As illustrated in Figure 16, between 8% and 43% of CD8 cells GFP+ indicating successful transduction.

Production of Target Cells expressing nf-P2X$_7$ or Wild-Type (WT) P2X$_7$ Receptors

[0253] To assess the efficacy of CD8 cells expressing the anti-nf-P2X$_7$-CARs, Hek293T cells over-expressing either a non-functional P2X$_7$ receptor (having a K193A mutation) or a wild type extracellular domain of the P2X$_7$ receptor on their cell surface, were prepared.

[0254] EXD2_K193A (nf-P2X$_7$) and EXD2_WT (functional P2X$_7$) gBlock gene fragments (SEQ ID NOs: 47 and 48, respectively) were ordered from Integrated DNA technologies (IDT). The EXD2 domains were designed to be expressed in frame with DNA sequences encoding for a fusion protein consisting of the IgK-leader-HA-MYC-PDGFR-transmembrane domian from pDisplay (Invitrogen - Figure 17). These fusion proteins were designed for surface expression. The EXD2_K193A and EXD2_WT gene fragments were cloned between the HA and MYC-epitope Tags to forma fusion gene blocks. Gateway attB1 and attB2

sequences were included at the 5'- and 3'- ends of the fusion gene blocks for cloning into the LV-416-IRES-puro vector (Clontech).

[0255] Cloning was performed using Gateway® (ThermoFisher) and all steps were carried out following the manufacturer's protocol. Briefly:

- First BP recombination reactions were performed between attB-flanked DNA fragments (EXD2_K193A, SEQ ID NO: 47 and EXD2_WT, SEQ ID NO: 48) and an attP containing pDONR-107 vector to generate an entry clone. The BP recombination reactions were used to transform chemically competent E.cloni®10G cells (Lucigen®) according to manufacturer's protocol;
- Transformed cells were plated onto LB agar plates containing 50ug/ml Kanamycin (Sigma) and incubated at 37 °C overnight;
- Two clones from each plate were picked to prepare mini cultures (2 mL) in LB broth with kanamycin (SIGMA) (50ug/ml). Followed by incubation at 37°C overnight with agitation.
- Plasmid DNA was extracted the following day from the mini cultures using the QIAGEN QuickLyse miniprep kit;
- A diagnostic *Bam H1*-HF (NEB) and *PmeI* (NEB) digests were performed to identify recombinant clones. Both the EXD2_K193A and EXD2_WT clones were confirmed to be digested correctly via *Bam H1* and *Bam H1/PmeI* digestion followed by gel electrophoresis (Figure 18).

[0256] One clone from each construct (EXD2_K193A and EXD2_WT) was chosen for the LR recombination reaction (as set forth below) to insert the EXD2_K193A and EXD2_WT constructs into a pLV-416 destination vector.

[0257] Following selection of clones, LR recombination reactions were then performed to transfer each EXD2 insert from the pDONR-107 entry clone to the pLV-416 destination vector to create an expression vector. The final LR recombination reaction was used to transform chemically competent E.cloni®10G cells (Lucigen®) according to manufacturer's protocol. Briefly:

- Transformed cells were plated onto LB agar plates containing 100ug/ml Ampicillin (SIGMA) and incubated at 37 °C overnight;
- Six clones from each plate were picked to prepare mini cultures (2 mL) in LB broth with Ampicillin (50ug/ml), which were incubated at 37 °C overnight with agitation;

- The following day plasmid DNA was isolated and *Bam H1* digestion was performed to identify recombinant clones. Recombinant clones were identified by the presence of three bands of the appropriate size (3431, 1056 and 5844 bp – see Figure 19). As can be seen in Figure 19 all six selected clones from each plate provided the appropriate sized restriction fragments;
- Two clones transduced with the pLV-416 constructs containing EXD2_K193A or EXD2_WT were sequenced with the primers set forth in Table 7 to confirm the constructs were correct.

TABLE 7

Primers Used for Confirmation of Correct EXD2_K193A and EXD2_WT Constructs in Selected Colonies

Primer name	Sequence	SEQ ID NO
EXD-F1 primer	ACAAGCTGTACCAGCGGAAA	SEQ ID NO: 49
EXD2-R1 primer	CACCACCACCTTAAAGGGCA	SEQ ID NO: 50
EXD2-F1 primer	ACAAGCTGTACCAGCGGAAA	SEQ ID NO: 51

[0258] To produce viral particles for transduction of HEK293 cells, and the generation of a stable HEK293 cell line, expressing a functional or non-functional $P2X_7$ receptor, the following protocol was used:

- HEK293 cells were plated ($7x10^6$ cells per flask) a day prior to transfection.
- HEK293T cells were transfected with Lentiviral packaging vectors and either pLV-416-EXD2 and pLV-416-EXD2_WT. To monitor transfection efficiency, a GFP expression plasmid (1ug) was also included.
- Following overnight incubation, the medium containing the transfection reagents was removed and replaced with 10 ml of fresh medium (DMEM with 10% FCS). 10 ml of media was harvested 24 hours later and stored in 2 ml aliquots at -80°C until required. Another 10 ml of fresh medium (DMEM with 10% FCS) was added to the flasks which was harvested a further 24 hours later.
- Viral particles were isolated from the harvested media by centrifuging the media at 1200 rpm followed by filtration through a 0.45um filter. The filtered media, with virus particles, were used for transfection of HEK293 cells.

[0259] To assess transfection efficiency, cells were harvested following the removal of the second 10ml of medium and the percentage of GFP positive cells was determined by flow

cytometry. Figure 20 illustrates that HEK293 cells were transfected with pLV-416-EXD2_K193A and pLV-416-EXD2_WT at an efficiency of 97% and 85%.

[0260] To generated stable HEK293 cells over-expressing the extracellular domain of functional and non-functional $P2X_7$ on their cell surface. The following protocol was used:

- HEK293 cells were plated ($7x10^5$ per flask) in T25 flasks a day prior to transduction.
- The following day the medium was removed from each flask, and fresh media containing virus particles produced in accordance with the above protocol were added according to the ratios set out in Table 8;
- Polybrene was added to each flask to a final concentration of 8 ug/mL.

Table 8

Transduction Protocol

Virus particle	Media	Media with virus	Media	Media with virus	Media	Media with virus
pLV-416-EXD2	2.5 mL	2.5 mL	4 mL	1 mL	4.5 mL	0.5 mL
pLV-416-EXD2_WT	2.5 mL	2.5 mL	4 mL	1 mL	4.5 mL	0.5 mL
Control LV-411-GFP	4 mL	1 mL (0.5 MOI)				
Polybrene 20 mg/mL stock	2 uL		2 uL		2 uL	
Un-transduced	5 mL					

- 24 h after the transduction, the medium was removed from each flask and fresh medium (DMEM with 10% FCS) supplemented with 1600 ug/mL G418 was added to all flasks except the flask containing control Lentivirus expressing GFP (LV-411-GFP).
- HEK293T cells transduced with control pLV-411-GFP virus were monitored for GFP expression 72 hours post-transduction (see Figure 21);
- All un-transduced cells died 4 days after culturing with G418 supplemented media. The transduced cell lines continued to grow normally with G418 in media.

[0261] The extracellular domain of the transfected $P2X_7$ receptors contains HA- and MYC-epitope tags. Therefore, these cells can be stained with monoclonal antibodies against HA- and MYC- to confirm surface expression of the extracellular domain by flow cytometry.

Screening for CAR T cell function

[0262] To assess the functionality of the nf-P2X7-CARs, CD8 cells transduced with each of

the PEP2-2-1-1 or PEP2-472-2 CAR constructs (as prepared above) were co-incubated for 4 hours in a 96-well round-bottom culture plate at 1:1 ratio with $1X10^4$ target cells expressing an nf-$P2X_7$ receptor (as prepared above) and MDA-MB-231 breast cancer cells, which express a non-functional $P2X_7$ receptor (231 $P2X_7$ cells).

[0263] The percentage of cytotoxicity was determined via a in a CytoTox 96® Non-Radioactive Cytotoxicity Assay (Promega, Madison, Wisconsin, USA) in accordance with the manufacturer's instructions. Briefly:

- 45 mins prior to 4 hours, 10µl of Lysis Solution (10X) was added to each well for every 100µl of target cells;
- After a further 45 minutes the plates were centrifuge at 250xg for 4 minutes;
- 50µl aliquots were taken from each well and transferred to a 96-well flat-bottom plate;
- 50µl of CytoTox 96® Reagent was added to each well of the plate containing the transferred aliquots, and the plate was cover with foil for 30 minutes at room temperature;
- Following 30 minutes, 50µl of Stop Solution was added to each well and the absorbance at 490nm was read from each well.

[0264] The absorbance values for each well was corrected in accordance with the manufacturer's instructions and the percentage of cytotoxicity was calculated using the following formula, normalised to empty vector transduced T cells, to give a fold change in cell killing.

$$\%\ \text{Cytotoxicity} = \frac{\text{Experimental} - \text{Effector Spontaneous} - \text{Target Spontaneous}}{\text{Target Maximum} - \text{Target Spontaneous}} * 100$$

[0265] As demonstrated in Figure 22A, both PEP2-2-1-1 and PEP2-472-2 CAR expressing CD8 T cell killed approximately 15 and 11-fold (respectively) more HEK cells expressing a non-functional $P2X_7$ receptor than CD8 cells transduced with an empty vector. Furthermore, as shown in Figure 22B, PEP2-2-1-1 and PEP2-472-2 CAR expressing CD8 T cell killed approximately 2.5 and 2.25-fold (respectively) more 231 $P2X_7$ cells than CD8 cells transduced with an empty vector.

[0266] All methods described herein can be performed in any suitable order unless indicated otherwise herein or clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., "such as") provided herein, is intended merely to

better illuminate the example embodiments and does not pose a limitation on the scope of the claimed invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential.

[0267] The description provided herein is in relation to several embodiments which may share common characteristics and features. It is to be understood that one or more features of one embodiment may be combinable with one or more features of the other embodiments. In addition, a single feature or combination of features of the embodiments may constitute additional embodiments.

[0268] The subject headings used herein are included only for the ease of reference of the reader and should not be used to limit the subject matter found throughout the disclosure or the claims. The subject headings should not be used in construing the scope of the claims or the claim limitations.

[0269] Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to, or indicated in this specification, individually or collectively, and any and all combinations of any two or more of the steps or features.

[0270] Also, it is to be noted that, as used herein, the singular forms “a”, “an” and “the” include plural aspects unless the context already dictates otherwise.

[0271] Future patent applications may be filed on the basis of the present application, for example by claiming priority from the present application, by claiming a divisional status and/or by claiming a continuation status. It is to be understood that the following claims are not intended to limit the scope of what may be claimed in any such future application.

CLAIMS

1. A chimeric antigen receptor including an antigen-recognition domain and a signalling domain, wherein the antigen-recognition domain recognises a dysfunctional $P2X_7$ receptor.

2. The chimeric antigen receptor according to claim 1, wherein the antigen-recognition domain recognises an epitope associated with an adenosine triphosphate (ATP)-binding site of the dysfunctional $P2X_7$ receptor.

3. The chimeric antigen receptor according to claim 1 or claim 2, wherein the dysfunctional $P2X_7$ receptor has a reduced capacity to bind ATP compared to an ATP-binding capacity of a wild-type (functional) $P2X_7$ receptor.

4. The chimeric antigen receptor according to any one of claims 1 to 3, wherein the dysfunctional $P2X_7$ receptor has a conformational change that renders the receptor dysfunctional.

5. The chimeric antigen receptor according to claim 4, wherein the conformational change is a change of an amino acid from a trans-conformation to a cis-conformation.

6. The chimeric antigen receptor according to claim 5, wherein the amino acid that has changed from a trans-conformation to a cis-conformation is proline at amino acid position 210 of the dysfunctional $P2X_7$ receptor.

7. The chimeric antigen receptor according to any one of claim 1 to 6, wherein the antigen-recognition domain recognises an epitope that includes proline at amino acid position 210 of the dysfunctional $P2X_7$ receptor.

8. The chimeric antigen receptor according to any one of claims 1 to 7, wherein the antigen-recognition domain recognises an epitope that includes one or more amino acid residues spanning from glycine at amino acid position 200 to cysteine at amino acid position 216 of the dysfunctional $P2X_7$ receptor.

9. The chimeric antigen receptor according to any one of claims 1 to 8, wherein the antigen-recognition domain comprises amino acid sequence homology to the amino

acid sequence of an antibody, or a fragment thereof, that binds to the dysfunctional $P2X_7$ receptor.

10. The chimeric antigen receptor according to any one of claims 1 to 9, wherein the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a fragment-antigen binding (Fab) portion of an antibody that binds to a dysfunctional $P2X_7$ receptor.

11. The chimeric antigen receptor according to any one of claim 9 or claim 10, wherein the antibody is a humanized antibody.

12. The chimeric antigen receptor according to any one of claims 1 to 9, wherein the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a single-chain variable fragment (scFv) that binds to a dysfunctional $P2X_7$ receptor.

13. The chimeric antigen receptor according to any one of claims 1 to 9, wherein the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a multivalent single-chain variable fragment (scFv) that binds to a dysfunctional $P2X_7$ receptor.

14. The chimeric antigen receptor according to claim 13, wherein the multivalent single-chain variable fragment (scFv) is a di-valent or tri-valent scFv.

15. The chimeric antigen receptor according to any one of claims 1 to 9, wherein the antigen-recognition domain includes amino acid sequence homology to the amino acid sequence of a single-antibody domain (sdAb) that binds to a dysfunctional $P2X_7$ receptor.

16. The chimeric antigen receptor according to any one of claims 1 to 15, wherein the signalling domain includes a portion derived from an activation receptor.

17. The chimeric antigen receptor according to claim 16, wherein the activation receptor is a member of the CD3 co-receptor complex.

18. The chimeric antigen receptor according claim 17, wherein the portion derived from the CD3 co-receptor complex is CD3-ζ.

19. The chimeric antigen receptor according to claim 16, wherein the activation receptor is an Fc receptor.

20. The chimeric antigen receptor according claim 19, wherein the portion derived from the Fc receptor is FcεRI or FcγRI.

21. The chimeric antigen receptor according to any one of claims 1 to 15, wherein the signalling domain includes a portion derived from a co-stimulatory receptor.

22. The chimeric antigen receptor according to any one of claims 1 to 21, wherein the signalling domain includes a portion derived from an activation receptor and a portion derived from a co-stimulatory receptor.

23. The chimeric antigen receptor according to claim 21 or claim 22, wherein the co-stimulatory receptor is selected from the group consisting of CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

24. A nucleic acid molecule including a nucleotide sequence encoding the chimeric antigen receptor according to any one of claims 1 to 23.

25. A nucleic acid construct including a nucleic acid molecule according to claim 24.

26. The nucleic acid construct according to claim 25, wherein expression of the nucleic acid molecule is under the control of a transcriptional control sequence.

27. The nucleic acid construct according to claim 26, wherein the transcriptional control sequence is a constitutive promoter.

28. The nucleic acid construct according to claim 26, wherein the transcriptional control sequence is an inducible promoter.

29. The nucleic acid construct according to any one of claims 25 to 28, further including an Internal Ribosome Entry Site (IRES).

30. The nucleic acid construct according to any one of claims 25 to 29, wherein the nucleic acid construct is a vector.

31. The nucleic acid construct according to claim 30, wherein the vector is a viral vector.

32. A genetically modified cell, the cell including the chimeric antigen receptor of any one of claims 1 to 23.

33. The genetically modified cell according to claim 32, wherein the cell includes two or more different chimeric antigen receptors.

34. A genetically modified cell, the cell including the nucleic acid molecule according to claim 24, or the nucleic acid construct according to any one of claims 25 to 31, or a genomically integrated form of the nucleic acid construct.

35. The genetically modified cell according to claim 34, wherein the nucleic acid molecule or the nucleic acid construct encodes two or more different chimeric antigen receptors.

36. The genetically modified cell according to claim 33 or claim 35, wherein the two or more different chimeric antigen receptors have different signalling domains.

37. The genetically modified cell according to any one of claims 32 to 36, wherein the cell includes a first chimeric antigen receptor with a signalling domain including a portion derived from an activation receptor and a second chimeric antigen receptor with a signalling domain including a portion derived from a co-stimulatory receptor.

38. The genetically modified cell according to claim 37, wherein the activation receptor is a member of the CD3 co-receptor complex or is an Fc receptor.

39. The genetically modified cell according to claim 37 or claim 38, wherein the co-stimulatory receptor is selected from the group consisting of CD27, CD28, CD30, CD40, DAP10, OX40, 4-1BB (CD137) and ICOS.

40. The genetically modified cell according to any one of claims 32 to 36, wherein the cell is further modified so as to constitutively express co-stimulatory receptors.

41. The genetically modified cell according to claim 40, wherein the cell is further modified so as to express ligands for the co-stimulatory receptors, thereby facilitating auto-stimulation of the cell.

42. The genetically modified cell according to any one of claims 32 to 41, wherein the cell is further modified to secrete cytokines.

43. The genetically modified cell according to claim 42, wherein the cytokines are selected from the group consisting of IL-2, IL-7, IL-12, IL-15, IL-17 and IL-21, or a combination thereof.

44. The genetically modified cell according to any one of claims 32 to 43, wherein the cell is a leukocyte.

45. The genetically modified cell according to any one of claims 32 to 44, wherein the cell is a Peripheral Blood Mononuclear Cell (PBMC).

46. The genetically modified cell according to any one of claims 32 to 45, wherein the cell is a lymphocyte.

47. The genetically modified cell according to any one of claims 32 to 46, wherein the cell is a T cell.

48. The genetically modified cell according to claim 47, wherein the T cell is a CD4+ T cell.

49. The genetically modified cell according to claim 47, wherein the T cell is a CD8+ T cell.

50. The genetically modified cell according to any one of claims 32 to 46, wherein the cell is a natural killer cell.

51. The genetically modified cell according to any one of claim 32 to 46 wherein the cell is a natural killer T cell.

52. A method of killing a cell expressing a dysfunctional $P2X_7$ receptor, the method including exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified cell having a chimeric antigen receptor, wherein the chimeric antigen receptor is directed against the dysfunctional $P2X_7$ receptor.

53. The method according to claim 52, wherein the chimeric antigen receptor directly recognises the dysfunctional $P2X_7$ receptor.

54. The method according to claim 52, wherein the chimeric antigen receptor recognises the dysfunctional $P2X_7$ receptor via an intermediate.

55. The method according to claim 54, wherein the intermediate is a probe that binds to a dysfunctional $P2X_7$ receptor and the chimeric antigen receptor recognises the probe.

56. The method of claim 52, further comprising the step of exposing the cell expressing a dysfunctional $P2X_7$ receptor to a probe.

57. The method according to claim 55 or claim 56, wherein the probe is an antibody or an aptamer.

58. The method according to any one of claims 55 to 57, wherein the probe includes a tag and the chimeric antigen receptor recognises the tag.

59. A method of killing a cell expressing a dysfunctional $P2X_7$ receptor, the method including exposing the cell expressing a dysfunctional $P2X_7$ receptor to a genetically modified cell according to any one of claims 32 to 51.

60. The method according to any one of claims 52 to 59, wherein the method further includes exposing the cell expressing a dysfunctional $P2X_7$ receptor to an exogenous cytokine.

61. The method according to any one of claims 52 to 60, wherein the genetically modified cell is a genetically modified cell autologous to the cell expressing a dysfunctional $P2X_7$ receptor.

62. The method according to any one of claims 52 to 61, wherein the cell expressing a dysfunctional $P2X_7$ receptor is within the body of a subject.

63. The method according to any one of claims 52 to 62, wherein the cell expressing a dysfunctional $P2X_7$ receptor is a cancer cell.

64. The method according to claim 63, wherein the cancer cell is selected from one or more of; brain cancer, oesophageal cancer, mouth cancer, tongue cancer, thyroid cancer, lung cancer, stomach cancer, pancreatic cancer, kidney cancer, colon cancer, rectal cancer, prostate cancer, bladder cancer, cervical cancer, epithelial cell cancers, skin cancer, leukaemia, lymphoma, myeloma, breast cancer, ovarian cancer, endometrial cancer and testicular cancer.

65. The method according to claim 63, wherein the cancer cell is selected from one or more of; lung cancer, oesophageal cancer, stomach cancer, colon cancer, prostate cancer, bladder cancer, cervical cancer, vaginal cancers, epithelial cell cancers, skin cancer, blood-related cancers, breast cancer, endometrial cancer, uterine cancer and testicular cancer.

66. The method according to any one of claims 63 to 65, wherein the cancer is metastatic.

67. The method according to any one of claims 63 to 66, wherein the cancer is stage III cancer.

68. The method according to any one of claims 63 to 66, wherein the cancer is stage IV cancer.

69. A method of expanding *in vitro* the genetically modified cell according to any one of claims 32 to 51, the method including the step of exposing the cell to an antigen for the chimeric antigen receptor.

70. The method according to claim 69, further including the step of exposing the cell to a cytokine.

71. A method of expanding *in vitro* the genetically modified cell according to any one of claims 32 to 51, the method including the step of exposing the cell to an antigen for the chimeric antigen receptor and simultaneously exposing the cell to a cytokine.

72. The method according to claim 70 or claim 71, wherein the cytokine is a member of the IL-2 subfamily, the interferon subfamily, the IL-10 subfamily, the IL-1 subfamily, the IL-17 subfamily or the TGF-β subfamily.

73. The method according to claim 70 or claim 71, wherein the cytokine is selected from the group consisting of IFN-γ, IL-2, IL-5, IL- 7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, TNF-α, TGF-β1, TGF-β2, TGF-β3 and GM-CSF, or a combination thereof.

74. A method of expanding *in vitro* the genetically modified cell according to any one of claims 32 to 51, the method including the step of exposing the cell to immobilised anti-CD3 and anti-CD28 antibodies.

75. A pharmaceutical composition including a genetically modified cell according to any one of claims 32 to 51 and a pharmaceutically acceptable carrier.

76. The pharmaceutical composition according to claim 75, further comprising a cytokine.

77. The pharmaceutical composition according to claim 76, wherein the cytokine is a member of the IL-2 subfamily, the interferon subfamily, the IL-10 subfamily, the IL-1 subfamily, the IL-17 subfamily or the TGF-β subfamily.

78. The pharmaceutical composition according to claim 76, wherein the cytokine is selected from the group consisting of IFN-γ, IL-2, IL-5, IL- 7, IL-8, IL-10, IL-12, IL-13, IL-15, IL-17, IL-18, TNF-α, TGF-β1, TGF-β2, TGF-β3 and GM-CSF, or a combination thereof.

79. The pharmaceutical composition according to any one of claims 75 to 78, further comprising an intermediate.

80. The pharmaceutical composition according to claim 79, wherein the intermediate is a probe that binds to a dysfunctional $P2X_7$ receptor and the chimeric antigen receptor recognises the probe.

81. The pharmaceutical composition according to claim 80, wherein the probe is an antibody or an aptamer.

82. The pharmaceutical composition according to claim 80 or claim 81, wherein the probe includes a tag and the chimeric antigen receptor recognises the tag.

FIGURE 1

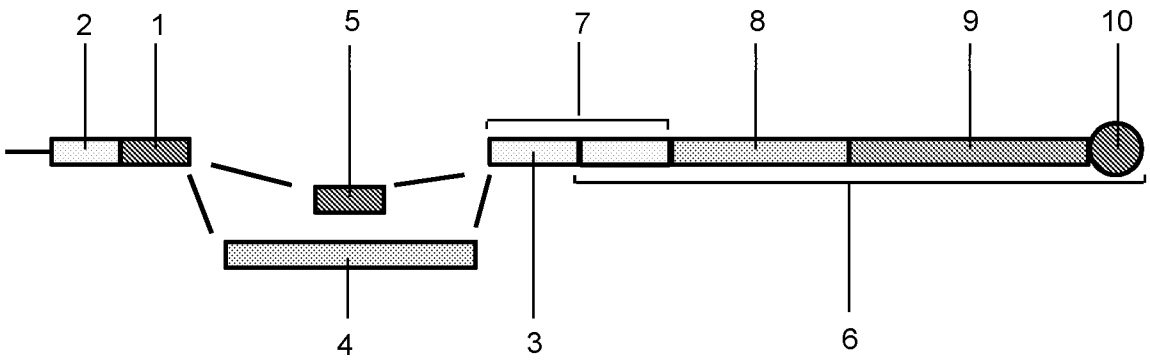


FIGURE 2

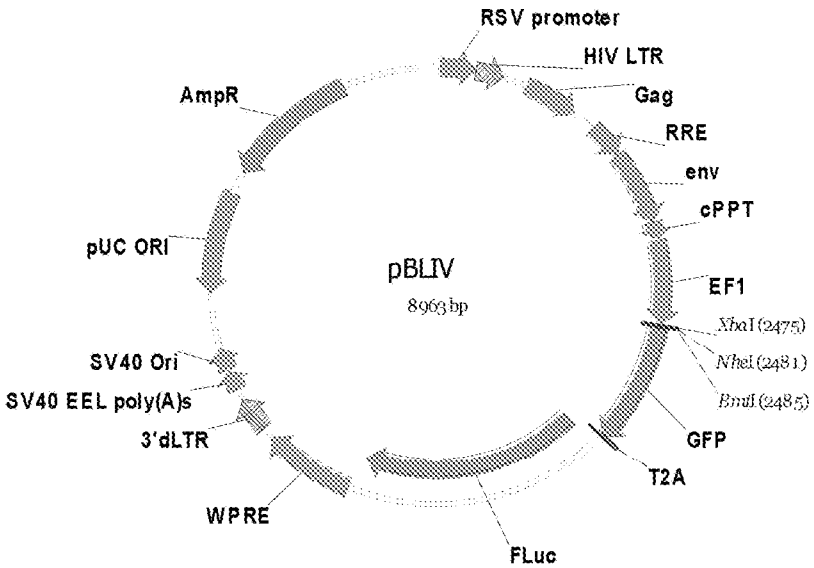
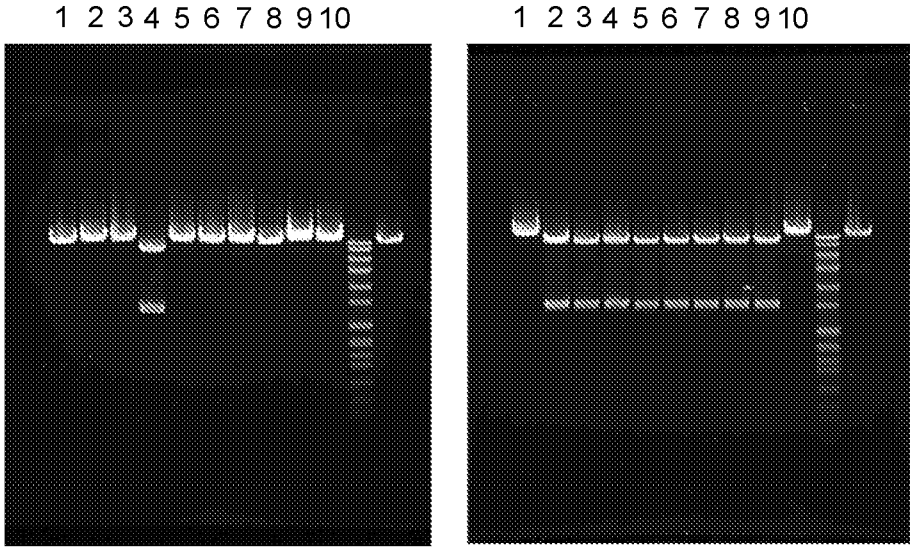


FIGURE 3



BLIV-CAR-short hinge BLIV-CAR-long hinge

FIGURE 4

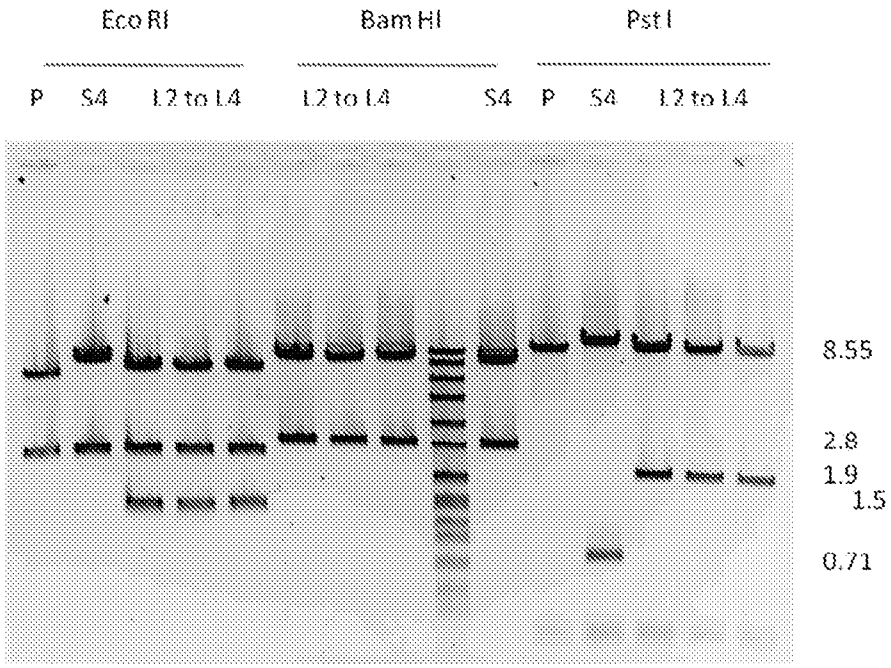
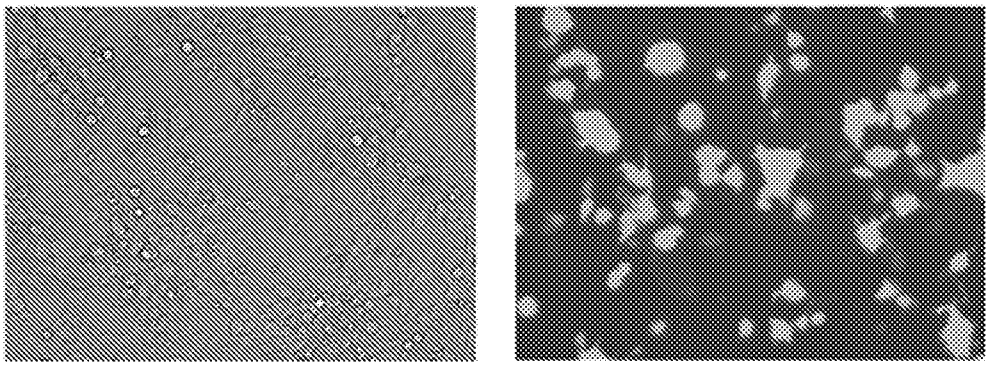


FIGURE 5

A

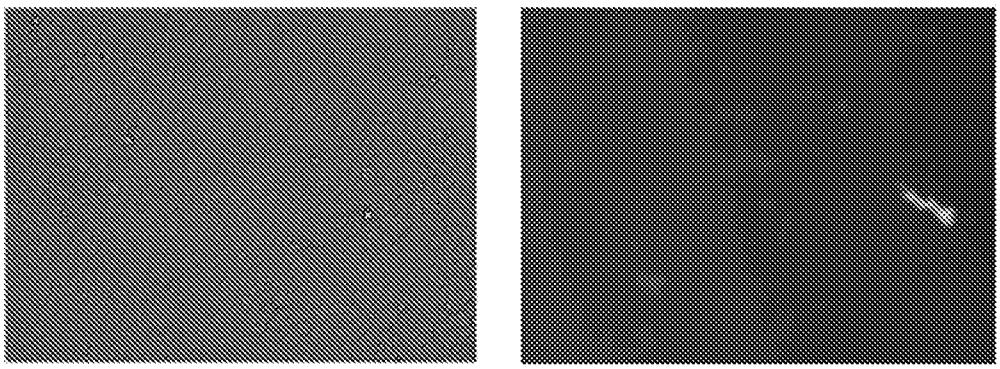
Transfection of 293T cell with BLIV-CAR-short hinge



Phase, 20x Green Fluorescence, 20x

B

Tranduction of 293T cell with BLIV-CAR-short hinge

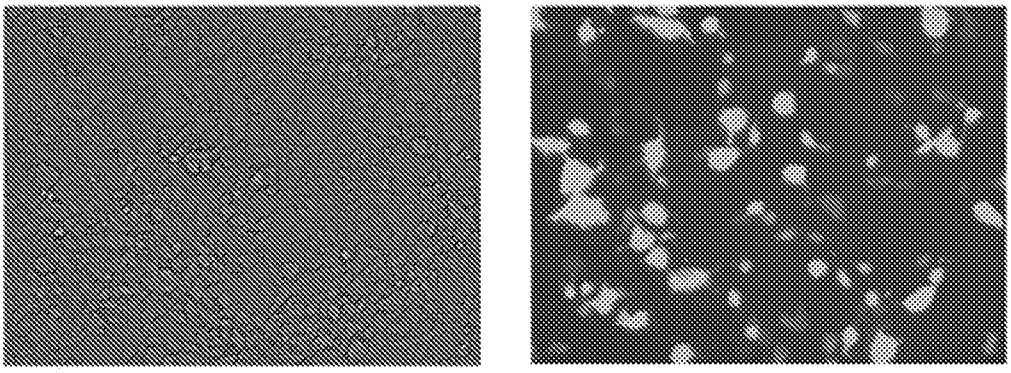


Phase, 20x Green Fluorescence, 30x

FIGURE 6

A

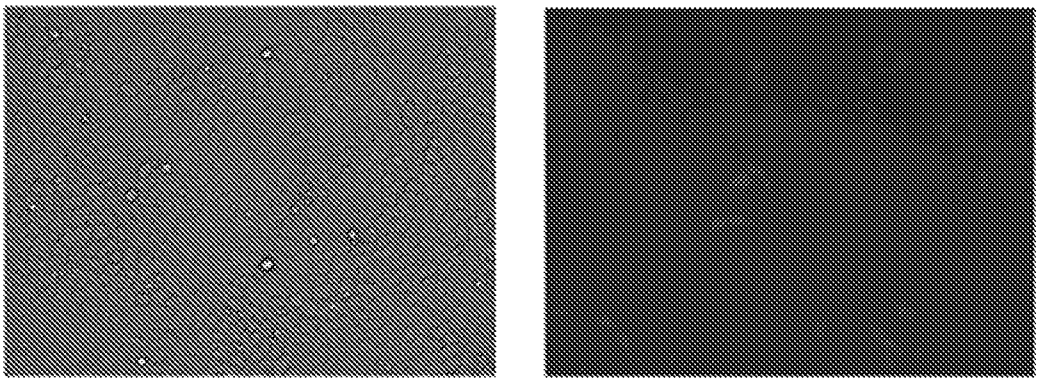
Transfection of 293T cell with BLIV-CAR-long hinge



Phase, 20x Green Fluorescence, 20x

B

Transduction of 293T cell with BLIV-CAR-long hing



Phase, 20x Green Fluorescence, 20x

FIGURE 7

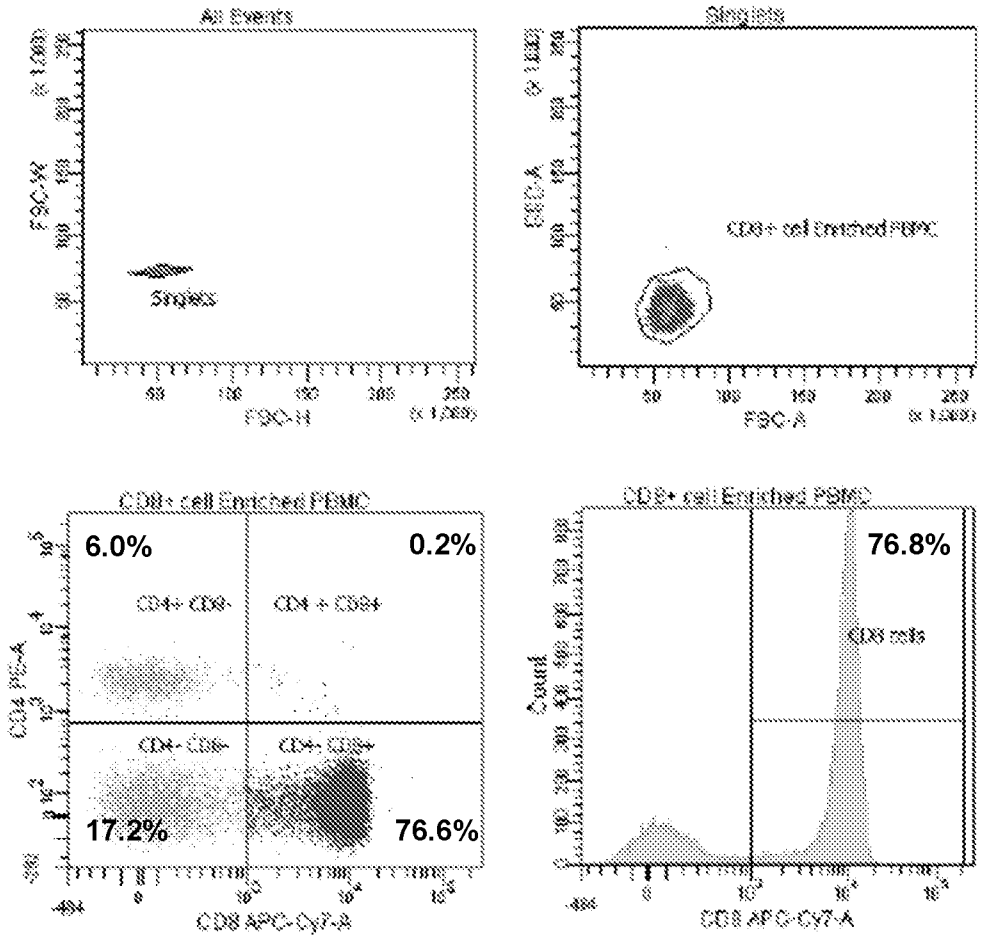

All Events
Singlets
FSC-H
FSC-A
CD8+ cell Enriched PBMC
6.0%
0.2%
17.2%
76.6%
CD4 PE-A
CD8 APC-Cy7-A
76.8%
Count

FIGURE 8

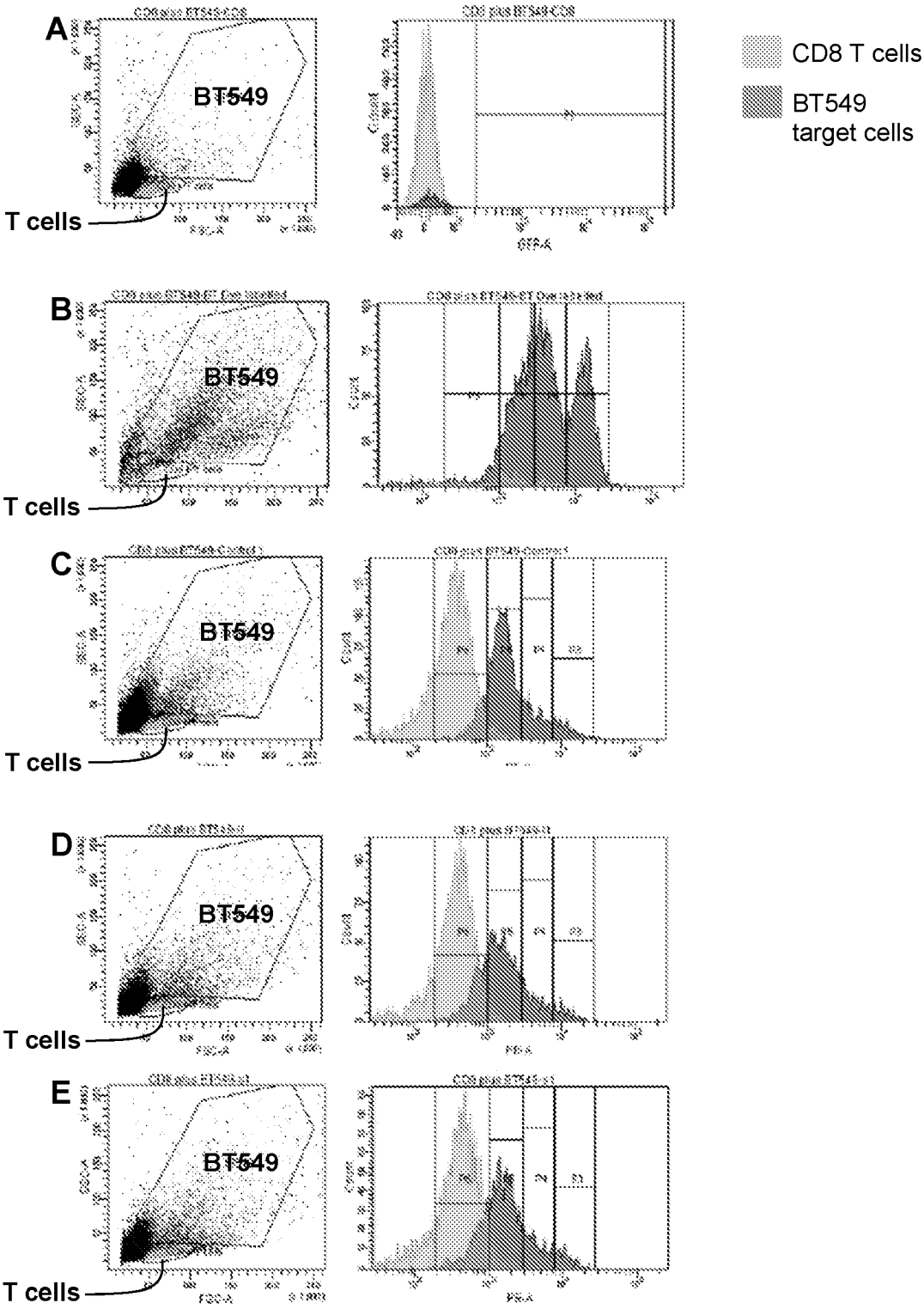

A
BT549
T cells
B
BT549
T cells
C
BT549
T cells
D
BT549
T cells
E
BT549
T cells
CD8 T cells
BT549 target cells

FIGURE 9

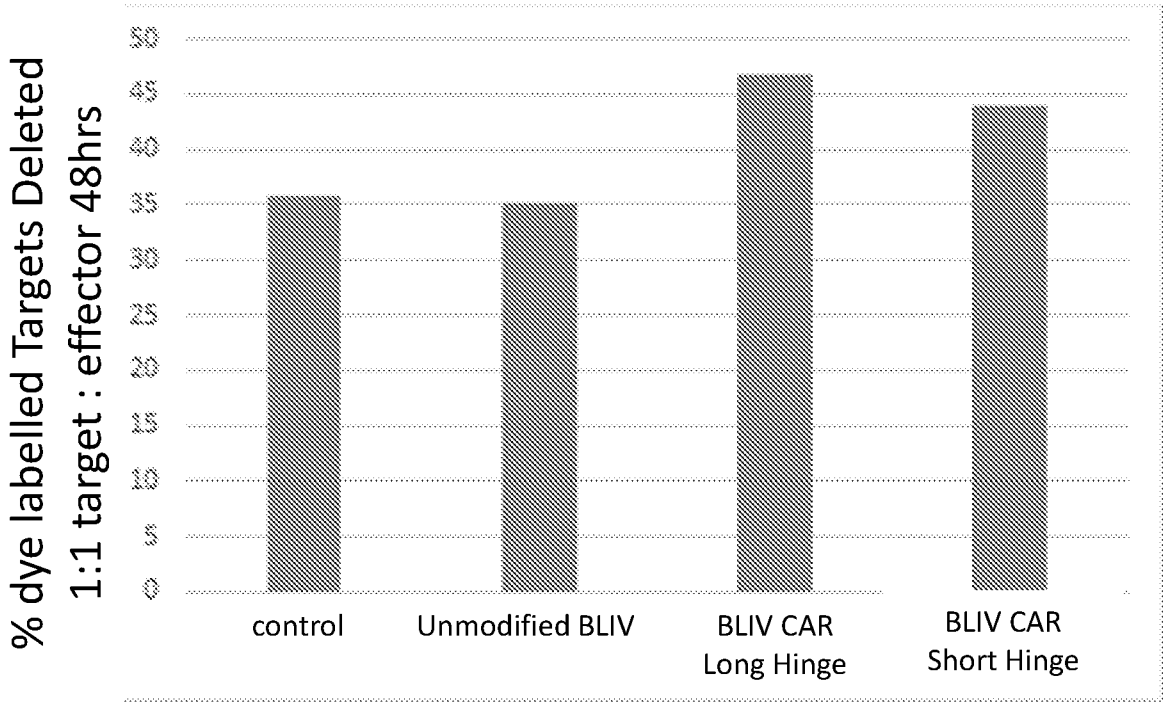

% dye labelled Targets Deleted
1:1 target : effector 48hrs
0
5
10
15
20
25
30
35
40
45
50
control
Unmodified BLIV
BLIV CAR
Long Hinge
BLIV CAR
Short Hinge

Figure 10

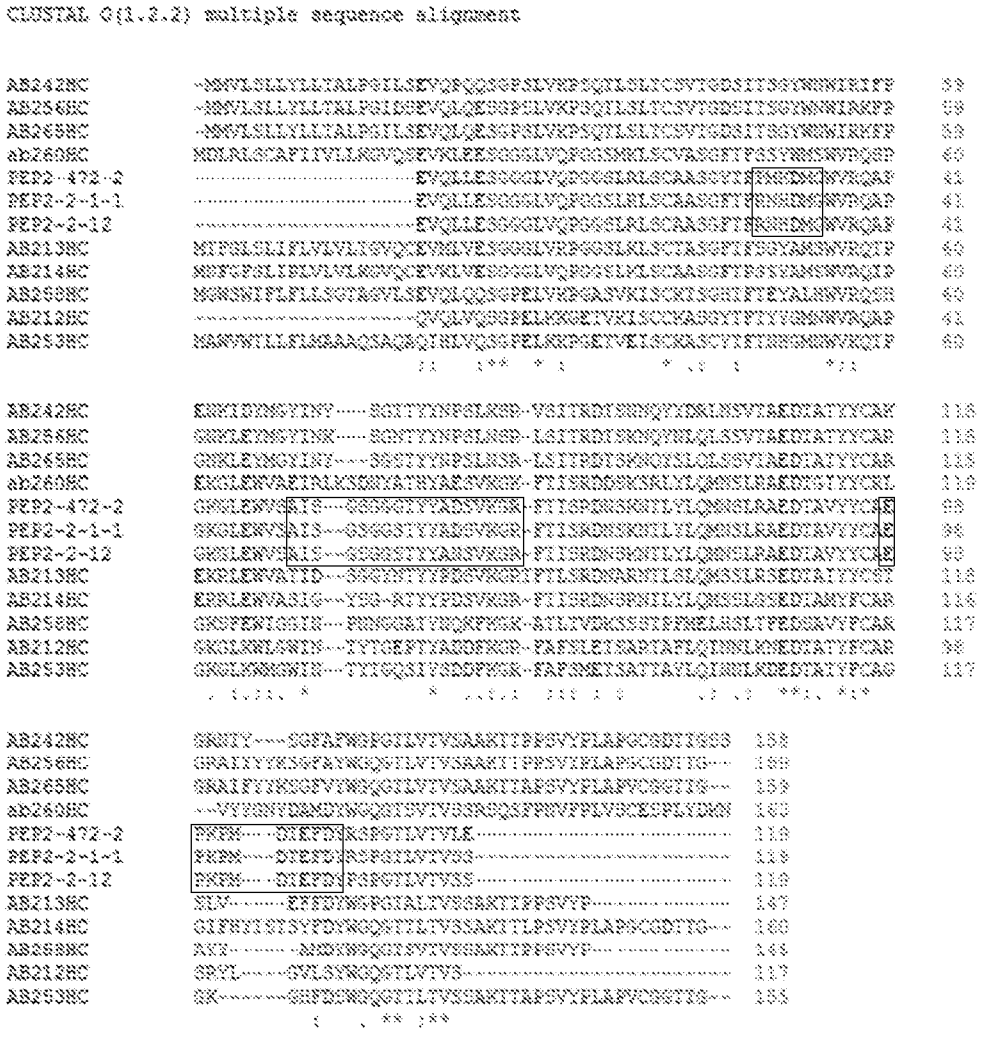


FIGURE 11

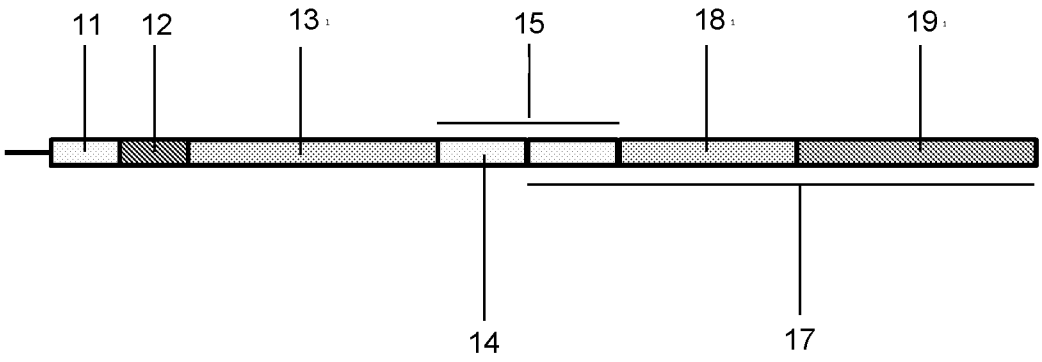


FIGURE 12

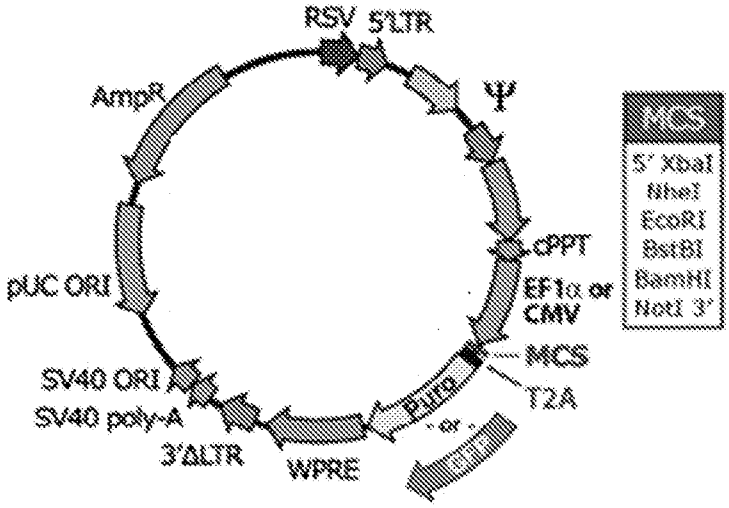


FIGURE 13

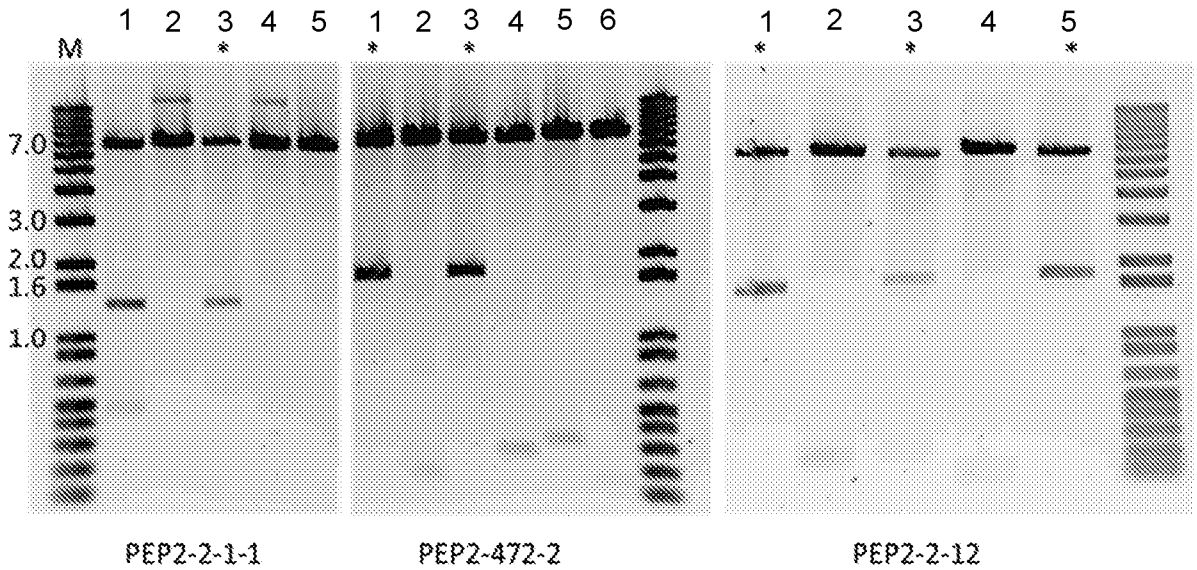


FIGURE 14

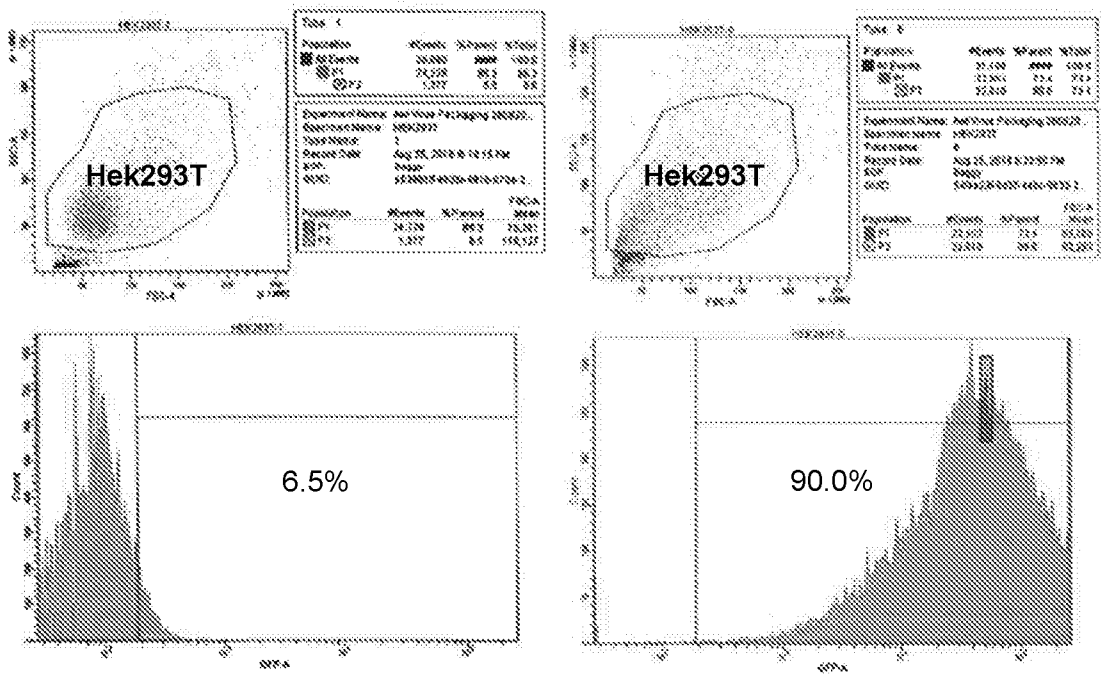


FIGURE 15

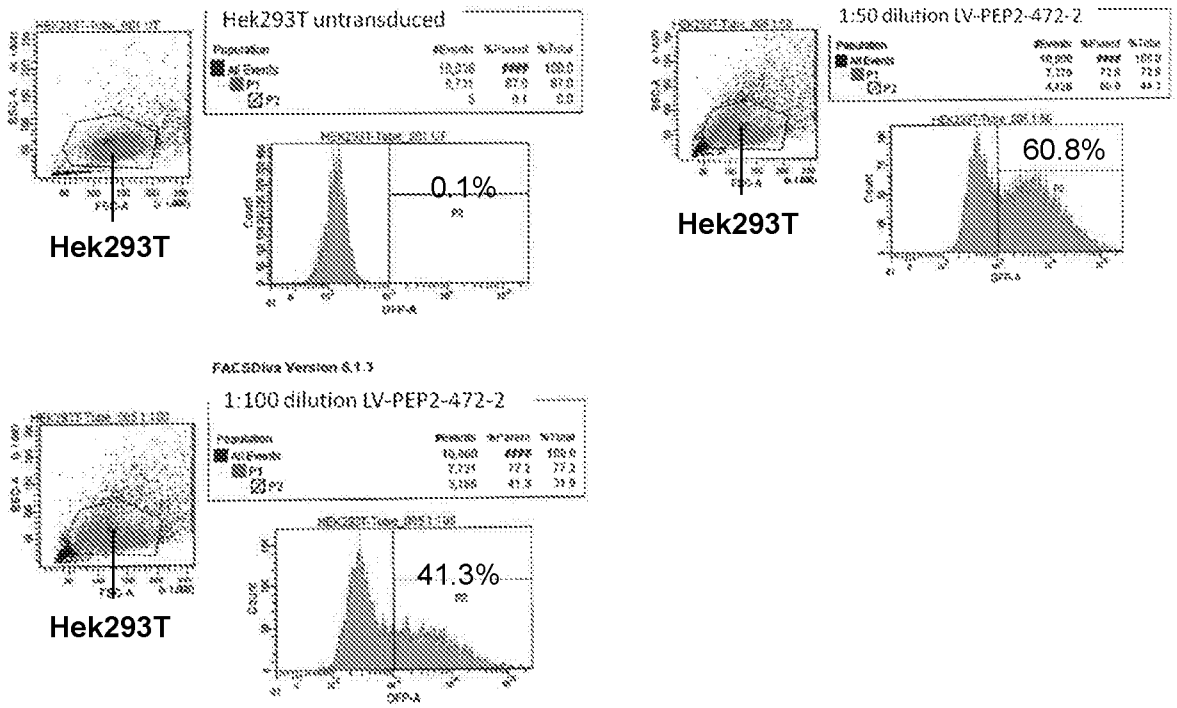


FIGURE 16

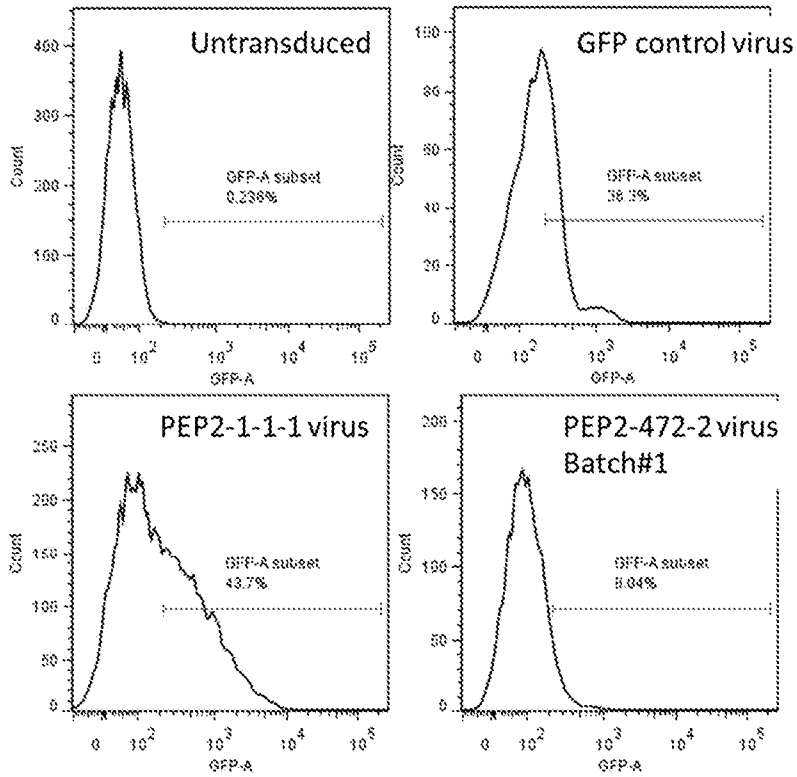


FIGURE 17

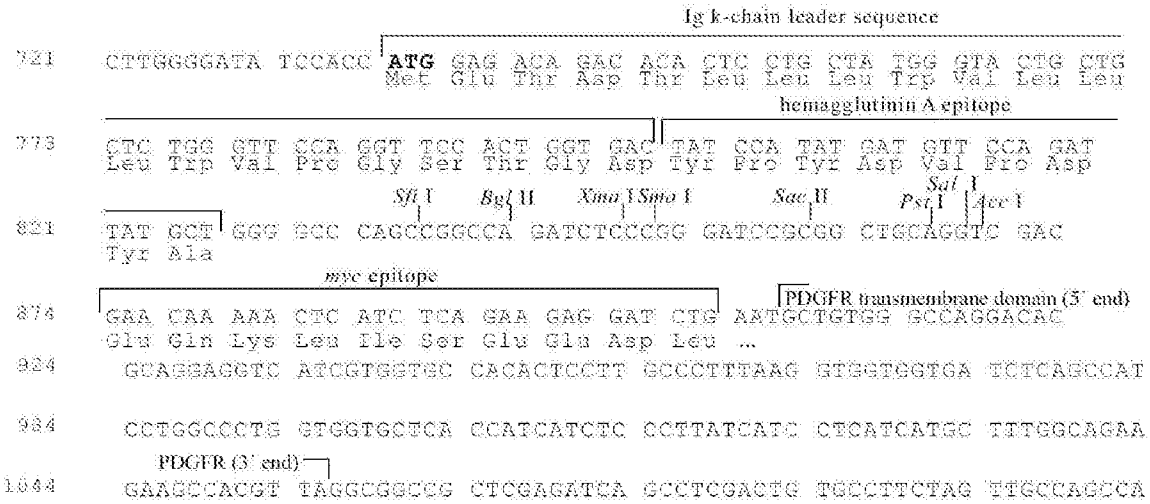


FIGURE 18

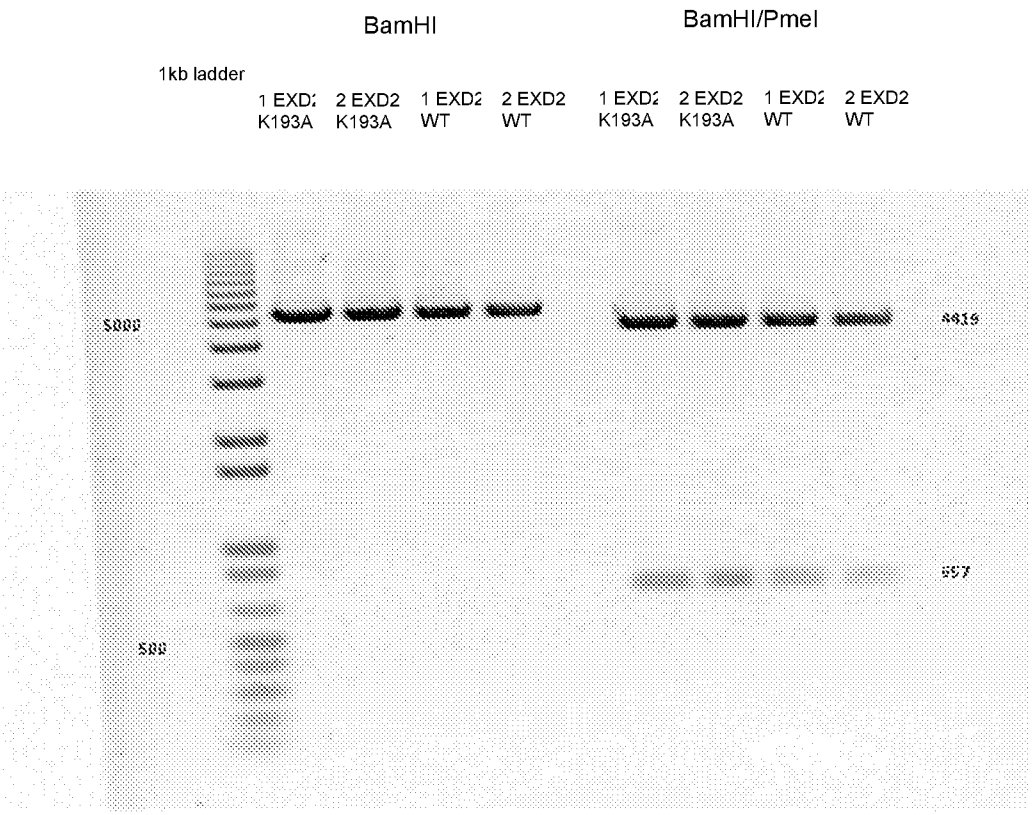


FIGURE 19

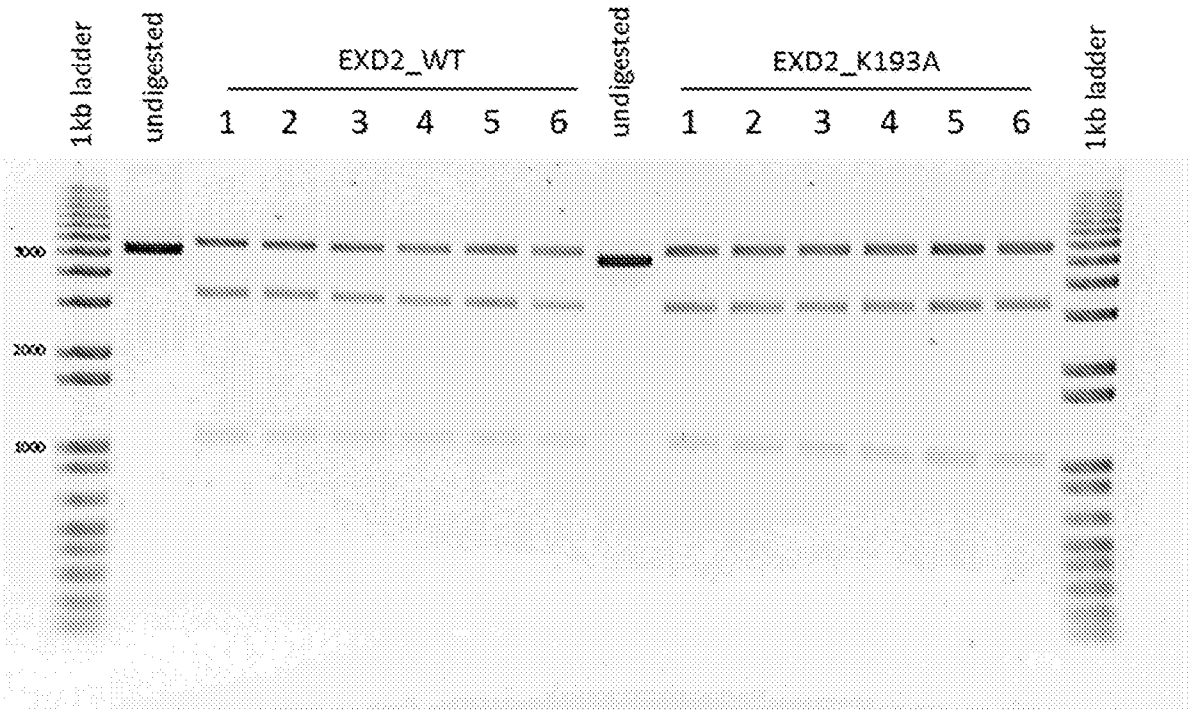


FIGURE 20

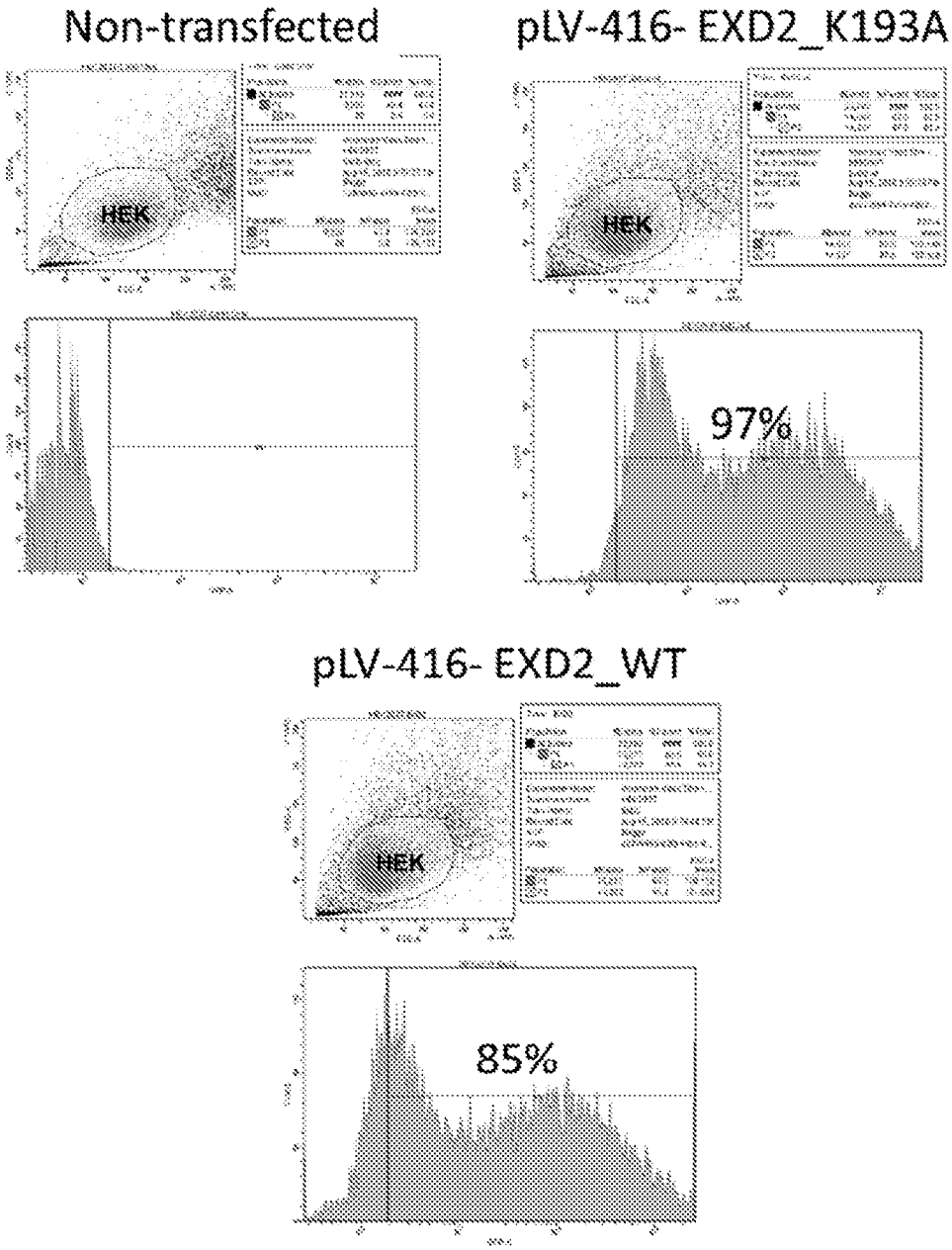

Non-transfected
HEK
pLV-416- EXD2_K193A
HEK
97%
pLV-416- EXD2_WT
HEK
85%

FIGURE 21

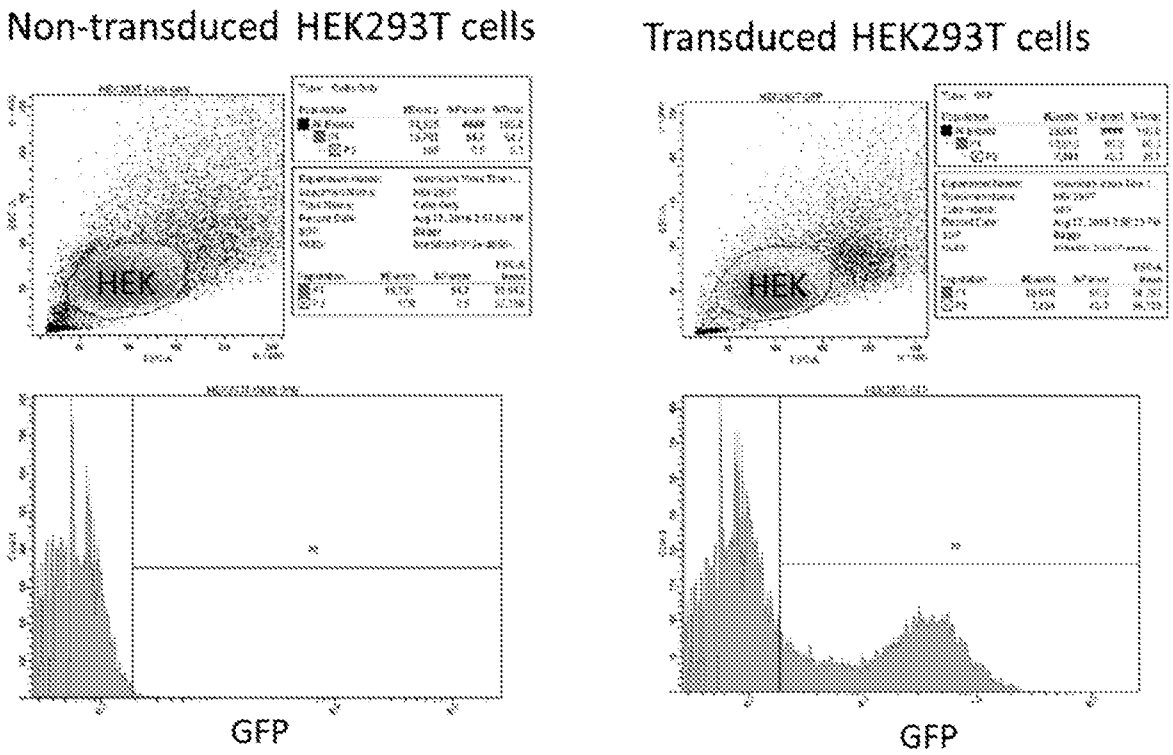
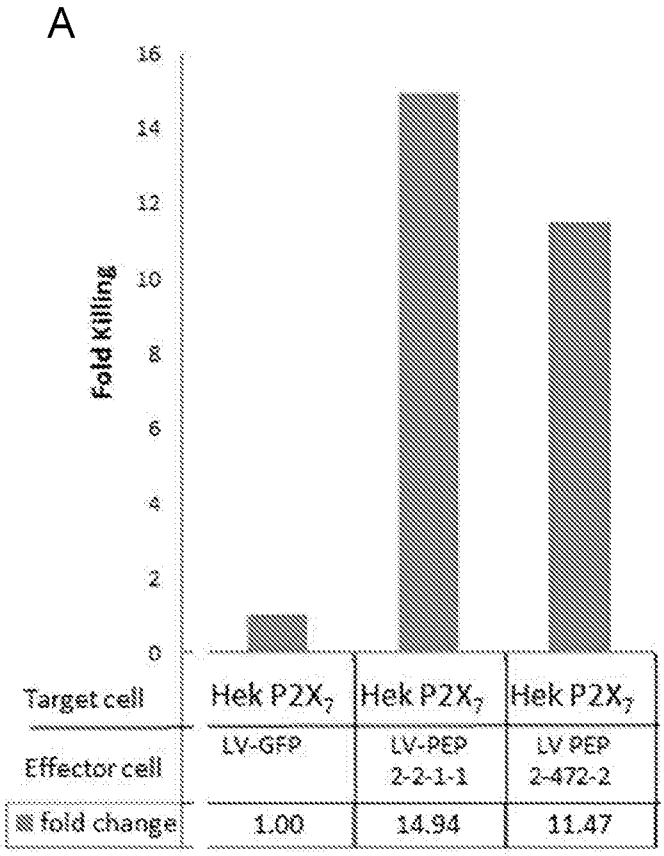
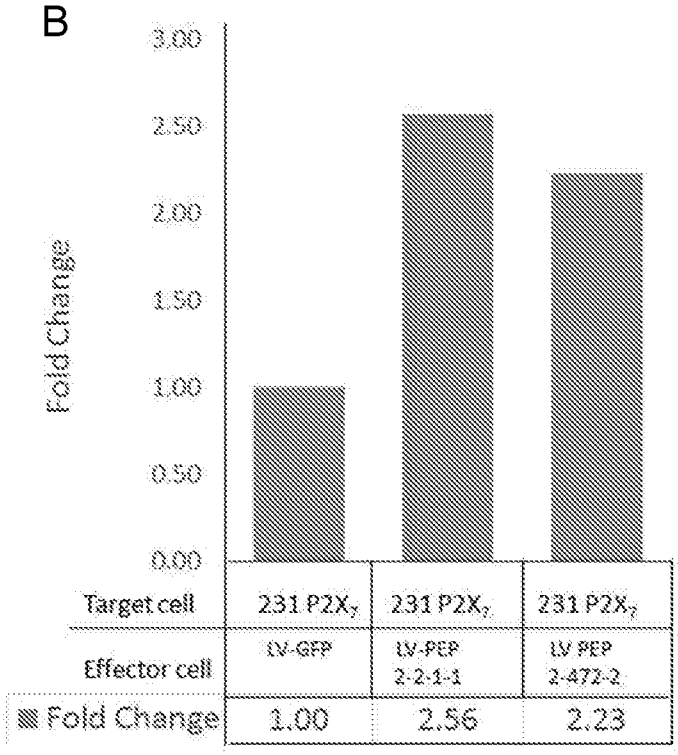

Non-transduced HEK293T cells
Transduced HEK293T cells
HEK
HEK
GFP
GFP

FIGURE 22

A


Fold Killing
16
14
12
10
8
6
4
2
0
Target cell
Hek P2X7
Hek P2X7
Hek P2X7
Effector cell
LV-GFP
LV-PEP 2-2-1-1
LV PEP 2-472-2
fold change
1.00
14.94
11.47

B


Fold Change
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Target cell
231 P2X7
231 P2X7
231 P2X7
Effector cell
LV-GFP
LV-PEP 2-2-1-1
LV PEP 2-472-2
Fold Change
1.00
2.56
2.23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050851

A. CLASSIFICATION OF SUBJECT MATTER

C12N 15/62 (2006.01) C12N 5/0781 (2010.01) C12N 5/0783 (2010.01) C07K 19/00 (2006.01) A61K 35/17 (2015.01) A61P 35/00 (2006.01)

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)

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)

WPIAP, EPODOC, EMBASE, MEDLINE, CAPLUS, BIOSIS; Keywords: chimeric antigen receptor, P2X7 receptor, nfp2x7, non-functional, pro210, and similar terms. GenomeQuest: amino acid sequences VLIKNNIDFPGHNYTTRNILPGLNITCTFHK and LYPGYNFRYAKYYKENNVEKRTLIKVFGIR (derived from SEQ ID NO: 1)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
	Documents are listed in the continuation of Box C	

[X] Further documents are listed in the continuation of Box C [X] 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
28 November 2016	28 November 2016
Name and mailing address of the ISA/AU	**Authorised officer**
AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA Email address: pct@ipaustralia.gov.au	Daniel Sheahan AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No. +61 2 6283 7969

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU2016/050851

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

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

   a. (means)

   ☐ on paper

   ☒ in electronic form

   b. (time)

   ☒ in the international application as filed

   ☐ together with the international application in electronic form

   ☐ subsequently to this Authority for the purposes of search

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

3. Additional comments:

INTERNATIONAL SEARCH REPORT C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		International application No. **PCT/AU2016/050851**
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2012/079000 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 14 June 2012 Abstract; page 2, lines 11-13; page 3, lines 3-5; page 14, lines 11-16; page 15, line 22 to page 16, line 3; page 27, line 30 to page 30, line 15; page 31, line 20 to page 37, line 10; page 44, line 10 to page 55, line 35	1-32, 34, 40-53, 59-78
Y	WO 2014/055657 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 10 April 2014 Abstract; page 13, lines 16-31; page 21; page 27, line 13 to page 35, line 14; page 37, line 26; page 43, line 1 to page 47, line 23; page 52 to page 53, line 12; Figure 1	1-53, 59-78
Y	WO 2011/075789 A1 (BIOSCEPTRE INTERNATIONAL LIMITED) 30 June 2011 Abstract; page 21, paragraph 2; pages 44 to 50; page 54, paragraphs 2-4; Examples 1 and 5	1-82
Y	WO 2011/020155 A1 (BIOSCEPTRE INTERNATIONAL LIMITED) 24 February 2011 Abstract; page 1, line 25 to page 2, line 3; page 9, lines 7-9, page 48, line 28 to page 50, line 7; page 70, line 14 to page 73, line 6; Example 1	1-82
Y	BARDEN, J.A. et al., "Non-functional P2X7: a novel and ubiquitous target in human cancer", Journal of Clinical & Cellular Immunology, 2014, Vol. 5:4 Abstract; page 2, paragraph 8; page 5, paragraphs 2 and 3	1-82
Y	WO 2012/082841 A2 (UNIVERSITY OF MARYLAND, BALTIMORE) 21 June 2012 Abstract; [0030], [0032]-[0040], [0043]-[0050], [0056]-[0063], Examples	52, 54-58, 60-68, 79-82
Y	STEPHAN, M.T. et al., "T cell-encoded CD80 and 4-1BBL induce auto- and transcostimulation, resulting in potent tumor rejection", Nature Medicine, 2007, Vol. 13, No. 12, pages 1440-1449 page 1441, paragraph 2 to page 1445, paragraph 1; page 1446, paragraph 1	40-43
Y	PERGRAM, H.J. et al., "Tumor-targeted T cells modified to secrete IL-12 eradicate systemic tumors without need for prior conditioning", Blood, 2012, Vol. 119, No. 18, pages 4133-4141 Abstract; page 4136, paragraph 3 to page 4137, paragraph 1	40-43
A	WO 2015/121454 A1 (CELLECTIS) 20 August 2015 Abstract; Table 6	
A	NESSELHUT, J. et al., "NfP2X7, a novel target for immune therapeutic approaches in cancer treatment", Journal of Clinical Oncology, 2013, Vol. 31, No. 15, May 20 Supplement, Abstract 3094	

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/050851**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		**Patent Family Member/s**	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
WO 2012/079000 A1	14 June 2012	WO 2012079000 A1	14 Jun 2012
		AU 2011338200 A1	04 Jul 2013
		CA 2820681 A1	14 Jun 2012
		CL 2013001645 A1	14 Feb 2014
		CN 103492406 A	01 Jan 2014
		CO 6801633 A2	29 Nov 2013
		CR 20130269 A	03 Sep 2013
		DO P2013000128 A	16 Mar 2014
		EA 201390847 A1	30 Dec 2013
		EC SP13012739 A	31 Oct 2013
		EP 2649086 A1	16 Oct 2013
		GT 201300150 A	10 Jun 2014
		JP 2014507118 A	27 Mar 2014
		JP 5947311 B2	06 Jul 2016
		JP 2016174607 A	06 Oct 2016
		KR 20130124521 A	14 Nov 2013
		MA 34813 B1	02 Jan 2014
		MX 2013006570 A	26 Aug 2013
		NZ 612512 A	27 Mar 2015
		PE 01782014 A1	20 Feb 2014
		SG 190997 A1	31 Jul 2013
		SG 10201510092Q A	28 Jan 2016
		TN 2013000246 A1	10 Nov 2014
		US 2013309258 A1	21 Nov 2013
		US 8906682 B2	09 Dec 2014
		US 2013288368 A1	31 Oct 2013
		US 8911993 B2	16 Dec 2014
		US 2014370017 A1	18 Dec 2014
		US 8916381 B1	23 Dec 2014
		US 2015050729 A1	19 Feb 2015
		US 8975071 B1	10 Mar 2015
		US 2015118202 A1	30 Apr 2015
		US 9101584 B2	11 Aug 2015
		US 2015093822 A1	02 Apr 2015
		US 9102760 B2	11 Aug 2015
		US 2015099299 A1	09 Apr 2015

Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

INTERNATIONAL SEARCH REPORT Information on patent family members	International application No. **PCT/AU2016/050851**

This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document/s Cited in Search Report		Patent Family Member/s	
Publication Number	**Publication Date**	**Publication Number**	**Publication Date**
		US 9102761 B2	11 Aug 2015
		US 2014106449 A1	17 Apr 2014
		US 9328156 B2	03 May 2016
		US 2016130355 A1	12 May 2016
		US 9464140 B2	11 Oct 2016
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Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.

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This Annex lists known patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

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