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(54) **ALL-IN ONE VECTOR FOR CAR AND THERAPEUTIC EFFECTOR MOLECULE**

VIRALER ALL-IN-ONE-VEKTOR FÜR CAR UND THERAPEUTISCHES EFFEKTORMOLEKÜL

VECTEUR VIRAL TOUT-EN-UN POUR MOLÉCULES CAR ET EFFECTRICES THÉRAPEUTIQUES

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- **KATHARINA ZIMMERMANN ET AL: "Design and Characterization of an "All-in-One" Lentiviral Vector System Combining Constitutive Anti-GD2 CAR Expression and Inducible Cytokines", CANCERS, vol. 12, no. 2, 6 February 2020 (2020-02-06), pages 375, XP055727985, DOI: 10.3390/cancers12020375**

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Description

[0001] The present invention relates to a vector for the constitutive expression of a second protein and the inducible expression of at least one first molecule, also referred to as a first protein, in mammalian cells. Generally, the vector contains an expression cassette for constitutive expression of the second protein and an expression cassette for inducible expression of the first molecule, wherein the second protein is a receptor molecule, e.g. a receptor, e.g. a CAR or a TCR, capable of binding a cognate antigen, and the first molecule is an effector molecule and expression of the first molecule is induced by binding of the second protein to its cognate antigen. The vector can be preferentially used for introduction and expression in immune cells, in particular lymphocytes, NK cells, and macrophages. The second protein and first molecule can be clinically relevant proteins. Accordingly, the vector is suitable for use in therapy, e.g. for use in immune therapy, especially in the treatment of cancer, including leukemia, lymphoma and solid tumours, or for use in immunomodulation, e.g. for use in the treatment of autoimmune diseases or for providing immune tolerance, e.g. immune tolerance for a cognate antigen. In a specific embodiment of the invention, the vector comprises a constitutive expression cassette encoding as the second protein a chimeric antigen receptor (CAR) or a T-cell receptor (TCR) and an inducible expression cassette encoding as the first molecule a therapeutic molecule, also referred to as effector molecule or as effector module, can e.g. be a peptide, protein, or RNA, which may be selected from shRNA, miRNA, lncRNA, and sgRNA. The therapeutic molecule can have stimulating activity on the immune response, e.g. the effector molecule can be a cytokine, lymphokine, chemokine, or tissue factor. Examples of effector molecules are IL-18, IL-12, IL-12 having a modified sequence, Il,15, IL15 having a modified sequence, IL21, IL2, TGF-beta, IL10, IL35, TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) and/or IFN-γ. The vector can be a viral vector comprising flanking LTR or SIN LTR, or flanking inverted terminal repeats (ITRs). Preferably, the vector is adapted for integration into the genome of an immune cell. Generally herein, a cognate antigen is also termed target antigen.

[0002] In a preferred embodiment of the invention, the viral vector is self-inactivating (SIN) upon integration into the DNA of a recipient cell. The vector has the advantages that the constitutive expression cassette for the second protein, which preferably is a CAR or a TCR, and the inducible expression cassette for the first molecule, which is also related to as effector molecule, in the absence of antigen binding to the CAR or TCR do not influence each other, i.e. the inducible allele only has very low background activity, and that both cassettes are present on a single vector, requiring only a single genetic modification to generate genetically manipulated immune cells. In the presence of antigen binding to the CAR or TCR, respectively, the first molecule is produced, i.e. as an antigen-specific response mediated by the signal transduction of the CAR or TCR.

State of the art

[0003] Chmielewski and Abken, Cell Reports 3205-3219 (2017), describe genetically manipulated T-cells that on the one hand contain a nucleic acid construct expressing a CAR that can bind to its target antigen through its specific single chain variable fragment (scFv) region, and on the other hand additionally contain a second nucleic acid construct for the cytokine IL-18 that either is inducible upon CAR engagement or constitutively. The additional inducible IL-18 vector construct consists of six NFAT elements followed by a minimal IL-2 promoter. IL-18 is solely expressed after antigen recognition of the CAR. Moreover, they also describe an IL-18 vector construct that constitutively expresses IL-18 independent of antigen recognition and engagement of the CAR construct.

[0004] Chmielewski and Abken, Cancer Immunol Immunother 1269-1277 (2012) describe the expression of IL-12 under the control of an NFAT-derived promoter upon binding of a CAR to its antigen, the CAR containing CD3ζ as an intracellular signalling domain. Kailayangiri et al., Oncoimmunology 2017, vol. 6, No. 1, e1250050, describe construction of a CAR that is specific for the antigen GD2.

[0005] WO 2019/051424 A2 describes genetic constructs which are transposons for constitutive expression of a Chimeric Antigen Receptor (CAR) and inducible expression of GFP or resp. of blood coagulation Factor IX.

[0006] WO 2018/132494 A1 describes vector constructs for the inducible expression of genes, wherein a DNA binding polypeptide and a transactivating polypeptide are connected by means of a linker.

[0007] WO 2019/006468 A1 describes DNA constructs for the constitutive expression of a CAR and the inducible expression of a prodrug cleaving enzyme, e.g. CPG2 or beta-lactamase.

[0008] US 2019/0112380 A1 describes genetically manipulated immune cells expressing chimeric antigen receptors and K13-vFLIP signalling protein for the treatment of cancer.

[0009] WO 2017/186121 A1 describes immune response cells constitutively expressing a chimeric antigen receptor and inducibly expressing exogenous interferon gamma.

Objective of the invention

[0010] The objective of the invention is to provide an alternative improved vector, especially a vector containing both

a CAR expression cassette and an additional inducible effector module which contains or is an expression cassette encoding RNA or a protein. This shall allow a specific increased expression of the effector molecule after CAR recognition of the cognate antigen. The effector can optionally be IL-18, IL-12 and/or another cytokine that can e.g. help to specifically modify the tumour microenvironment and recruit other immune cells. Preferably, the vector shall be suitable for immunomodulation, e.g. for induction of tolerance or treatment of autoimmunity via e.g. immunomodulatory effector modules like Il,10 or TGF-beta. Preferably, the viral vector shall enable the production of a high titer of viral particles which is suitable for clinical applications. Further preferred, the invention shall provide T-cells transduced by the vector, optionally other immune cells transduced by the vector, for use in the treatment of tumour or of virally infected cells. Preferably, the inducible cytokine expression should be tightly linked to the CAR expression and shall not be active without any presence of the target antigen.

Description of the invention

[0011] The invention achieves the objective by the features of the claims, especially by a nucleic acid construct, herein also generally referred to as a vector or viral vector, and by immune cells containing the nucleic acid construct, especially for use in immune therapy, e.g. for use in the treatment of cancer, or for use in the treatment of autoimmune disease, or for use in the treatment of GvH or HvG. The nucleic acid construct comprises a second expression cassette for constitutive expression of a CAR or a TCR, the binding of which to its target or cognate antigen results in signalling and induces the expression of an effector molecule from a first expression cassette, which is contained on the same nucleic acid construct, and which first expression cassette encodes the effector molecule under the control of a promoter inducible by signalling of the CAR or TCR. Therein, the second molecule is a receptor molecule for a cognate antigen and the first molecule is an effector molecule, and the nucleic acid construct is set up to express the effector molecule upon signalling by the CAR or TCR binding to its cognate antigen. The nucleic acid construct preferably contains integration elements, e.g. viral LTR elements and the vector is e.g. a lentiviral, an alpha (α)-retroviral or a gamma (γ)-retroviral vector. Further, the integration elements can be transposase recognition sites. The nucleic acid construct can optionally be operably linked to a promoter controlling transcription for use in production of viral particles that contain the viral vector, wherein the production is in eukaryotic host cells. The nucleic acid construct, or vector, comprises or consists of at least one expression cassette for a first molecule, herein also referred to as an effector molecule, and at least one expression cassette for a second protein, herein generally represented by a CAR, which expression cassettes are arranged between a 5'LTR and a 3'SIN LTR. In embodiments, in which the at least one expression cassette for an effector molecule and the at least one expression cassette for a CAR are arranged on the same strand of one nucleic acid construct, i.e. forming one vector, more preferred on one common RNA or DNA strand, the expression cassette for an effector molecule is also referred to as a first expression cassette, which is arranged in 5' to the expression cassette for the CAR, which is also referred to as a second expression cassette. It was found that this vector architecture is very important, because another arrangement of these expression cassettes results in readthrough, and can result in unwanted background expression of the effector molecule. An arrangement of both cassettes in antisense orientation leads to RNA interference decreasing vector titers. It was found further that the orientation of the promoter of the first expression cassette and of the coding sequence of the effector molecule in sense-orientation, i.e. sense orientation of the first expression cassette, and sense orientation of the second expression cassette is preferable. Accordingly, it is preferred that the first expression cassette and the second expression cassette are on the same strand, DNA or RNA, and that the first expression cassette is in 5' to the second expression cassette. In contrast to a high induction for sense orientation of both expression cassettes, antisense orientation of the first expression cassette resulted in decreased expression of the sense-oriented expression cassette encoding the CAR. Accordingly, it is preferred that the first expression cassette is arranged directly adjacent to the second expression cassette, wherein both expression cassettes are arranged on one nucleic acid strand, so that both the expression cassettes are arranged in the same direction of transcription, i.e. in sense orientation. Further preferred, a packaging signal (Ψ element) is arranged between the 5' SIN LTR and the first promoter of the first expression cassette

[0012] Upon integration of the nucleic acid construct, e.g. in an embodiment of a viral vector, into the genome of an immune cell, e.g. by transduction with viral particles containing the vector, the LTR (long terminal repeat) which is arranged in 5' of the at least one first expression cassette and the at least one second expression cassette, is replaced by a copy of the SIN LTR (self-inactivating LTR) that is arranged in 3' to the first and second expression cassettes, e.g. at the 3' terminus of the vector, resulting in a genome-integrated and non-replicating copy of the vector. In the nucleic acid construct, the first and the second expression cassettes may be arranged between two SIN LTR elements. Accordingly, the vector can contain two SIN LTR, flanking the first and the second expression cassettes.

[0013] The viral vector can be an alpha retroviral SIN vector, a gamma retroviral SIN vector, or a lentiviral SIN vector.

[0014] Preferably, a viral packaging signal (Ψ element) is arranged between at least one of the LTR or of the transposase recognition sites and an expression cassette, more preferably, a Ψ element is arranged between a 5' LTR, respectively a 5' transposase recognition site, and the adjacent expression cassette, which preferably is the first expression cassette.

[0015] The arrangement of the expression cassettes on one strand, DNA or RNA, with the first expression cassette in 5' to the second expression cassette in the vector has the advantage of being one nucleic acid construct that integrally contains and is sufficient for providing an immune cell with both a CAR or TCR directed against a target antigen, and with an expression cassette encoding an effector molecule, wherein the expression of the effector molecule is only inducible by binding of the CAR or TCR to its target antigen, preferably with significantly lower or preferably no expression of the effector in the absence of target antigen for the CAR or TCR. This All-in-One configuration of the vector enables simplified clinically translatable production processes comprising the alteration, e.g. exchange of the antigen binding domain of the CAR or TCR, and production of only one vector construct, and provides therefore an advantage compared to current combinations of vectors, in which one separate vector construct is used for CAR expression and a second vector construct is used for expression of an effector module. The process produces immune cells for use in the treatment of cells that express the target antigen of the CAR or TCR, respectively. The cells expressing the target antigen can e.g. be tumour cells expressing a tumour antigen as the target antigen, immune cells that cause an autoimmune disease, e.g. immune cells expressing an MHC molecule as the target antigen, which MHC molecule recognizes a self-antigen, or for use in the treatment of HvG disease in transplant patients, wherein the target antigen is e.g. an MHC molecule of the graft. The process can be an in vitro process, using autologous immune cells that originate from the later patient to be treated, and the process can comprise a step of controlling immune cells into which a nucleic acid construct of the invention has been introduced, and selecting immune cells which contain the nucleic acid construct of the invention, preferably selecting immune cells which contain the nucleic acid construct of the invention and excluding cells having tumour markers.

[0016] Further, viral particles containing the one nucleic acid construct according to the invention, also termed All-in-One vector, containing or consisting of both the first expression cassette and the second expression cassette, preferably flanked by LTR sites, LTR and SIN-LTR sites, can be produced at high titer in a production cell. For production of viral particles containing the vector, preferably a split-packaging system is used, which e.g. comprises or consists of the vector, a first helper plasmid expressing the viral structural proteins and replication enzymes (gag-pol), and a second helper plasmid for expression of the retroviral envelope protein (env). For lentiviral vector production, an additional rev protein is expressed that binds to the Rev response elements (RRE) and facilitates packaging and the export of transcripts. For production, especially of nucleic acid constructs of the invention which are viral vectors, cultivated human or non-human mammalian cells are preferred. For nucleic acid constructs that contain transposase recognition sites, e.g. ITR, that flank the first expression cassette and the second expression cassette, the nucleic acid construct can be in combination with transposase, e.g. encoded on a nucleic acid section that is arranged on a section that is not flanked by the transposase recognition sites, e.g. ITR, or the transposase can be provided as an addition plasmid. The region encoding the transposase preferably is an expression cassette for transposase, having a promoter in 5' to the transposase encoding region and a poly-adenylation signal in 3' to the transposase encoding region. For genetic manipulation of an immune cell, both the nucleic acid construct containing the first expression cassette and the second expression cassette and the region encoding the transposase are transfected into the immune cell.

[0017] Furthermore, the vector has the advantage that once the target antigen is present, the binding of the CAR to its target antigen directly results in the induction of the expression of the effector molecule from the first expression cassette, whereas in the absence of the target antigen a significantly lower or no expression of the effector molecule occurs. This shows that the vector is set up to express the effector molecule encoded by the first expression cassette essentially only in response to the presence of the target antigen, for which the CAR is specific. This behaviour of the vector is highly desirable, because the effector molecule is only produced by cells, preferably immune cells, containing the vector in the presence, i.e. in contact with the target antigen, and hence the effector is produced only in the vicinity of e.g. cells bearing the target antigen. Accordingly, the vector, respectively immune cells containing the vector, are suitable for use in the medical treatment of cells expressing the target antigen, and especially of solid tumours, of autoimmune disease, or for providing tolerance, by generating a cellular immune response directed against cells bearing the target antigen in combination with secreting an effector molecule in the vicinity only of cells bearing the target antigen. For use in the treatment of a tumour, preferably of a solid tumour, the target antigen can be a tumour antigen, and the CAR is specific for the tumour antigen. For use in the treatment of an autoimmune disease, the target antigen can be the antigen against which the autoimmune disease is directed, and the CAR is specific for the target antigen.

[0018] Due to the localized cytokine secretion in the presence of the target antigen only, e.g. at the site of inflammation, e.g. IL-12 or IL-18 as effector molecule, this vector has the advantage to shape the tumour microenvironment by recruiting other immune cells, e.g. macrophages, dendritic cells or NK cells, and to therefore improve the proinflammatory immune and antitumour responses. Based on the modular architecture of the vector, it is also possible to express other effector molecules, e.g. other cytokines, e.g. IL-15, that are known to prolong the survival and persistence of the immune cells and thus directly affect the immune response. In this regard, the vector can be used to influence the immune response in an antiinflammatory manner, expressing as effector molecule e.g. IL10 and/or TGF-beta, e.g. for use in the treatment of GvH disease or in HvG disease in order to prolong transplant survival, or for use in the treatment of an autoimmune disease.

[0019] As immune cells containing the vector are set up to secrete the effector encoded by the first expression cassette only in the presence of the target antigen of the CAR, it was found that essentially no systemic secretion of the effector molecule occurs, avoiding adverse systemic side effects of the effector. In detail, the constitutively expressed CAR of the second expression cassette activates expression of the effector molecule encoded by the inducible first expression cassette through its intracytoplasmatic effector domain, and therefore activates the secretion of the immune effector molecule directly in the tissue bearing the target antigen recognized by the CAR, e.g. within the tumour, and due to this localized expression of effector molecules, which e.g. induce inflammation, possible adverse systemic side effects are minimized or avoided. This direct functional link between the CAR binding its target antigen and expression of the effector molecule provides an improved control of both the genetically modified target cells and the inducible secretion of the effector molecule and represents an additional advantage of the all-in-one vector system. Moreover, the use of just one vector reduces the risk of insertional mutagenesis and of cooperative oncogenesis. Finally, a reduction of steps for genetic manipulation of immune cells to a transduction with just a single All-in-One vector viral particles is more practical and e.g. allows use of clinical processes for producing for effector-enhanced CAR/TCR T-cells, NK cells and other immune cells.

[0020] The effector molecule can be selected e.g. from IL-12, IL-15, IL-18, IL10, TGF-beta, IL-2, IL-21 and IFN-γ. Preferably, IL-12 is encoded by SEQ ID NO: 4, which in human immune cells has been found to be expressed more efficiently than the wild-type coding sequence, and IL-18 is preferably encoded by SEQ ID NO: 6, which in human immune cells has been found to be expressed more efficiently than the wild-type coding sequence. For IL12, the chains are preferably directly fused to one another.

[0021] The vector can be contained in various immune cells, e.g. T-cells, preferably primary T cells (also including primary regulatory T cells), primary NK cells, primary NKT cells, macrophages, NK92 cells and dendritic cells. The immune cell containing the vector, respectively the immune cell which is genetically manipulated to contain the vector, can be a T-cell, a primary NKT-cell, a NK92 cell, a macrophage, or a dendritic cell, preferably a primary T-cell. Preferably, for the first promoter being the NFκBenhsyn promoter, the immune cell is a primary NK cell or a NK92 cell. More detailed, the immune cell originates from the patient who is the later recipient of the immune cell containing the vector.

[0022] Alternatively, immune cells with downregulated MHC and/or downregulated TCR expression, which are e.g. obtainable as pre-fabricated cells, can be genetically manipulated to contain the vector.

[0023] In embodiments, in which immune cell is a NK cell, especially when the vector is an alpha-retroviral or lentiviral vector, the first promoter preferably is the NFκBenhsyn promoter as a stronger promoter, or the first promoter is the NFAT promoter as a weaker promoter.

[0024] The CAR, from N-terminus to C-terminus, generally comprises or consists of an antigen binding domain - optionally a hinge - transmembrane domain - intracytoplasmatic effector domain, wherein the binding domain is a target antigen-binding domain, e.g. a scFv, a nanobody, a darpin, a ligand, or the extracellular portion of a T-cell receptor or of a B-cell receptor. The intracytoplasmatic effector domain preferably comprises or consists of the CD3zeta (CD3ζ) domain or e.g. the FceRI g-chain, with or without at least one co-stimulating domain, e.g. selected from the CD28 domain, the 41BB domain and the CD27 domain, preferably the intracytoplasmatic effector domain from N-terminus to C-terminus consists of the CD3ζ domain and an adjacent CD28 domain. In the presence of target antigen binding to the binding domain, at least a portion of the intracytoplasmatic effector domain, e.g. the CD3ζ domain, dimerizes. The co-stimulating domain may enhance the activating effect of the CAR when binding target antigen. In an alternative to CD3ζ, e.g. for NK cells, the intracytoplasmatic effector domain of the CAR can be the intracellular effector domain of DAP12 or DAP10. In the preferred embodiments, the CAR, which can be a T-cell receptor (TCR), as its intracellular signalling domain comprises a CD3ζ domain, which directly or mediated or assisted by transcription factors of eukaryotic cells, activates promoters containing an NFAT element or an NFκBenhsyn element, especially a promoter of one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 31.

[0025] For production of viral particles containing the nucleic acid construct containing or consisting of both the first expression cassette and the second expression cassette, in a production cell the nucleic acid construct preferably is under the control of a promoter selected from the group of the Cytomegalovirus (CMV) promoter (SEQ ID NO: 12), the Rous sarcoma virus (RSV) promoter (SEQ ID NO: 13), or the HIV U3 promoter (SEQ ID NO: 14). In case of gammaretroviral vectors also MLV-derived promoters (e.g. the SFFV or MPSV promoter) may be used. The vector has the advantage for clinical application that it can be produced at high titers that are sufficient for the generation of genetically modified T cells and other immune cells, e.g. NK cells and NK T cells, under good manufacturing practice (GMP) conditions. It was found that by the exchange of the RSV promoter to the CMV promoter within the 5' LTR region the titer of viral particles containing the vector in producer cells was increased to the 10-fold.

[0026] According to the invention, the first expression cassette from 5' to 3' comprises or consists of a first promoter containing a TATA box, e.g. in the minimal IL2 promoter or enhanced element, a coding sequence encoding the effector molecule, and optionally in 3' adjacent to the coding sequence a terminator or preferably is devoid of a terminator. Preferably, these elements are arranged directly adjacent to one another. The first promoter is inducible by the host cell containing the vector and is induced by NFAT (nuclear factor of activated T-cells) and preferably has SEQ ID NO: 1,

also referred to as NFATenhsyn promoter, because it has shown enhanced induction, e.g. effecting higher expression of the coding sequence than a known wild-type NFAT promoter, and it is a synthetic promoter element. As an alternative, the first promoter can have SEQ ID NO: 2 (NFATmIL2) and consists of an minimal IL2 promoter including a TATA box and fused to six NFAT response elements, or SEQ ID NO: 3 (NfκBenhsyn), where the NFAT response elements were exchanged for two NFκB response elements, or SEQ ID NO: 31. It was found that the first promoter having

SEQ ID NO: 1 (NFATenhsyn)

(TGGAGGAAAAACTGTTTCATACAGAAGGCGTGGAGGAAAAACTG
TTTCATACAGAAGGCGTGGAGGAAAAACTGTTTCATACAGAAGGCGTGGAGGAA
AAACTGTTTCATACAGAAGGCGTGGAGGAAAAACTGTTTCATACAGAAGGCGTG
GAGGAAAAACTGTTTCATACAGAAGGCGTCTGCAGGAGACTCTAGAGGGTATAT
AATGGTTTAAACTTAAGCTTGGTACCGGGCCCCCGAAG),

SEQ ID NO: 2 (NFATmIL2)

(GATATCGAATTAGGAGGAAAAACTGTTTCATACAGAAGGCGTCAA
TTAGGAGGAAAAACTGTTTCATACAGAAGGCGTCAATTAGGAGGAAAAACTGTT
TCATACAGAAGGCGTCAATTGGTCCCATCGAATTAGGAGGAAAAACTGTTTCATA
CAGAAGGCGTCAATTAGGAGGAAAAACTGTTTCATACAGAAGGCGTCAATTAGG
AGGAAAAACTGTTTCATACAGAAGGCGTCAATTGGTCCCGGGACATTTTGACACC
CCCATAATATTTTTCCAGAATTAACAGTATAAATTGCATCTCTTGTTCAAGAGTTC
CCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCCTG) ,

or
SEQ ID NO: 3 (NfκBenhsyn)

(CTCGAGGGGAATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTT
CCGGGAATTTCCGGGAATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTTCCG
GGAATTTCCCTGCAGGAGACTCTAGAGGGTATATAATGGTTTAAACTTAAGCTTG
GTACCGGGCCCCCGAAG),

or
SEQ ID NO: 31 (nucleotides 4178..4400 of SEQ ID NO: 32) (NfκBmIL2)

GGGAATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTTCCGGGAATTTCCGGG
AATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTTCCGGGAATTTCCCCCGGG
ACATTTTGACACCCCCATAATATTTTTCCAGAATTAACAGTATAAATTGCATCTCT
TGTTCAAGAGTTCCCTATCACTCTCTTTAATCACTACTCACAGTAACCTCAACTCC
TG

results in a high expression of the effector, whose expression is only induced in the presence of the target antigen of the CAR. It is generally preferred that in 3' of the first promoter having SEQ ID NO: 1 and in 5' to the coding sequence, a TATA box is arranged, which TATA box preferably has sequence 5' - T A T A (A/T) A (A/T) - 3' and respectively a nucleotide sequence of nucleotides No. 202-208 of SEQ ID NO: 1 or a nucleotide sequence of nucleotides 291-297 of SEQ ID NO: 2 or a sequence of nucleotides 131-137 of SEQ ID NO: 3. In embodiments, in which the first promoter has SEQ ID NO: 2 or SEQ ID NO: 3, the coding sequence can be arranged directly adjacent to the first promoter. Preferably, the first promoter is selected from the group comprising or consisting of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 31.

[0027] The second expression cassette from 5' to 3' comprises or consists of a second promoter, which is a constitutive promoter, a coding sequence encoding a CAR, optionally a WPRE (woodchuck hepatitis virus posttranscriptional regulatory element, preferably SEQ ID NO: 15), optionally in 3' adjacent to the WPRE a terminator or preferably no terminator. Preferably, these elements are arranged directly adjacent one another. Preferably, the constitutive second promoter is a PGK (phosphoglycerokinase) promoter, e.g. having a nucleotide sequence of nucleotides 5158..5673 of SEQ ID NO: 8.

[0028] A poly-adenylation signal is contained in the 3' LTR, preferably a 3' SIN LTR, e.g. in its R region with U5. There is no poly-adenylation signal between the first expression cassette and the second expression cassette. Generally, there is no poly-adenylation signal between the second expression cassette and the 3' SIN LTR. It has been found that a poly-adenylation signal arranged between the flanking LTRs results in a decrease in titer of viral particles in production cells. Accordingly, it is generally preferable for viral vectors of the invention, e.g. nucleic acid constructs containing LTR and SIN LTR flanking the first and second expression cassettes, that between flanking LTR and SIN LTR, between flanking ITRs, the nucleic acid construct is devoid of a poly-adenylation signal. In embodiments containing transposase recognition sites flanking the first and second expression cassettes, it is preferred that in 5' to the 3'ITR, e.g. directly in 3' to the second expression cassette, there is arranged an additional poly-adenylation signal.

[0029] The PGK promoter is the preferred promoter for the second expression cassette encoding the CAR, because the PGK promoter was found not to activate neighbouring genes, i.e. does not have a cis-activating effect when the vector is integrated into the genome of an immune cell. Similarly, for example the EF-1 alpha short (EFS, SEQ ID NO: 11) promoter, when arranged in the second expression cassette, was found also not to induce transcription from the first expression cassette encoding the effector. In contrast to PGK and EFS promoters, the spleen focus forming virus (SFFV, SEQ ID NO: 10) U3 promoter when used as the second promoter for the CAR showed higher transactivation of the first expression cassette. Accordingly, promoters with weak transactivation potential, like the PGK promoter and the EFS promoter, are preferred over a strong promoter with transactivation potential, e.g. SFFV.

[0030] The invention is now described in greater detail and with reference to the figures, wherein

- Fig. 1 shows an embodiment of the arrangement of the elements of a viral vector according to the invention,
- Fig. 2 shows an embodiment of the arrangement of the elements of a viral vector according to the invention,
- Fig. 3 shows expression levels from vectors in T-cells after CAR-specific stimulation,
- Fig. 4A shows results for production of viral particles containing a vector of the invention,
- Fig. 4B shows expression levels of an effector from T cells containing the vector after CAR-specific stimulation,
- Fig. 4C shows cytotoxicity results for immune cells (T cells) containing the vector,
- Fig. 5 in A, C, and E shows schematic representations of vectors, and in B, D, and F shows FACS results for expression of the effector from vectors without induction, with induction, and with unspecific induction,
- Fig. 6A shows expression of the effector in T cells transduced with alpharetroviral all-in-one vectors after CAR-specific stimulation, and
- Fig. 6B shows an embodiment of the arrangement of the elements of a viral vector after exchange of the NFAT element to the NFκB element,
- Fig. 6C shows the comparison between the NFATenhsyn and NFκBenhsyn promoters in alpharetrovirally transduced NK92 cells and its effector induction after CAR-specific stimulation,
- Fig. 7A schematically shows two embodiments of a viral vector according to the invention encoding hIL21 and hTRAIl, as the effector molecules, respectively,
- Fig. 7B shows secretion levels of hIL21 and Fig. 7C shows secretion levels of hTRAIL, resp., of primary T-cells containing a vector shown in Fig. 7A with or without contact with glioblastoma cells that express the target antigen for the CAR,
- Fig. 8A, 8B, 8C and 8E show bar graphs of expression of a representative effector molecule for different first promoters from immune cells harbouring the vector and stimulated by presence of the target antigen of the CAR,
- Fig. 8D shows FACS results for EGFP as a representative effector molecule under control of different first promoters without (w/o) and with stimulation by cells expressing the target antigen GD2 for the GD2-specific CAR.

[0031] In the Figures, the exemplary CAR, which is specific for GD2, is also designated as GD2CAR. Fig. 1 and Fig. 2 schematically show exemplary nucleic acid constructs of a first expression cassette 1 and a second expression cassette

2 of the invention between a 5' LTR and a 3' SIN LTR. As generally preferred, the first expression cassette 1 is arranged in 5' of the second expression cassette 2, and both the first and the second expression cassettes 1, 2 are on the same strand of one nucleic acid construct.

[0032] Fig. 1 shows two embodiments, in which the first expression cassette 1 consists of a promoter of SEQ ID NO: 1 (NFATenhsyn), a TATA box of nucleotides 202..208 of SEQ ID NO: 1, and a coding sequence for the effector, represented by eGFP (EGFP) or a cytokine (Cytokine), and the second expression cassette 2 consists of the PGK promoter (PGK), a coding sequence for a CAR having an antigen-binding domain (CAR of interest), and a WPRE (wPRE). In two further embodiments shown in Fig. 2, the first expression cassette 1 consists of the NFATmIL2 promoter (NFATmIL2) of SEQ ID NO: 2 directly adjoined by a coding sequence for the effector, exemplified by EGFP or a cytokine (Cytokine). The second expression cassette 2 is the same as in the other two embodiments. Neither the first expression cassette 1, nor the second expression cassette 2 contains an internal terminator in 3'of the coding sequence. Generally, the 3'LTR, more specifically the R region with U5, can serve as the polyadenylation signal.

Example 1: Expression of effector and of CAR from lentiviral vectors

[0033] As a representative for an effector, the coding sequence for eGFP (enhanced green fluorescent protein, EGFP) or the coding sequence for IL-12 (IL12) was contained in a first expression cassette under the control of the NFATenhsyn promoter (NFATenhsyn) having SEQ ID NO: 1 or under the control of the NFATmIL2 promoter having SEQ ID NO: 2, and the second expression cassette, arranged in 3' to the first expression cassette, contained a GD2-specific CAR (GD2CAR) under the control of the PGK promoter (PGK, nucleotides 5158..5673 of SEQ ID NO: 8), followed by the WPRE (SEQ ID NO: 15, PRE). A GD2-specific CAR (GD2CAR, e.g. as described by Kailayangiri et al., Oncoimmunology 2017) contained, from its N-terminus to its C-terminus, an antigen-binding domain specific for the exemplary target antigen Ganglioside 2 (GD2), a hinge, a transmembrane domain, a 4-1BB signalling domain fused to a CD3ζ domain. The first and the second expression cassettes were flanked by a 5' LTR and a 3' SIN LTR.

[0034] The vector encoding eGFP (EGFP) under the control of the NFATenhsyn promoter is designated pCCL.PPT.NFATenhsyn.EGFP.PGK.CAR.wPRE (SEQ ID NO: 8), wherein the CAR was a GD2-specific CAR.

[0035] The vector which in the alternative to the NFATenhsyn promoter in the first expression cassette contained the promoter having SEQ ID NO: 2 (NFATmIL2) in 5' to the effector (eGFP) encoding portion was designated pCCL.PPT.NFATmII,2.EGFP.PGK.CAR.wPRE (SEQ ID NO: 20), wherein the CAR was a GD2-specific CAR. An alpha retroviral vector construct having the NFκBmIL2 promoter in the first expression cassette is shown in SEQ ID NO: 26.

[0036] As a further example, a vector having the same elements except for the first expression cassette encoding human IL-12 (IL12human) instead of eGFP was used, this vector was designated pCCL.PPT.NFATenhsyn.humanIL12.PGK.CAR.wPRE (SEQ ID NO: 16) for the first expression cassette containing the NFATenhsyn promoter, wherein the CAR was a GD2-specific CAR. A vector containing the promoter NFATmIL2 of SEQ ID NO: 2 for the first expression cassette was designated pCCL.PPT.NFATmIL2.humanIL12.PGK.CAR.wPRE (SEQ ID NO: 22), wherein the CAR was a GD2-specific CAR. A vector containing the promoter NFκBmII,2 of SEQ ID NO: 31 for the first expression cassette was designated pCCL.PPT.NFκBmII,2.EGFP.PGK.CAR.WPRE (SEQ ID NO: 32), wherein the CAR was the GD2-specific CAR. Herein, pCCL designates a lentiviral transfer vector containing chimeric CMV-HIV 5' LTRs. In pCCL, the enhancer and promoter of CMV were joined to the R region of HIV-1. The vector constructs designated pCAS are alpha-retroviral vectors having alpha-retroviral LTRs.

[0037] For transduction of lentiviral, gamma- or alpha-retroviral particles containing the vector, primary human T-cells were isolated from human peripheral blood mononuclear cells (PBMC) from buffy coats. The T cells were activated with αCD3/αCD28 antibodies before transduction and were then transduced with the viral particles using RetroNectin (available from TaKaRa) as an enhancer of transduction.

[0038] The primary human T-cells containing one of the vectors were cultivated in RPMI medium containing 10% FCS (fetal calf serum), 1% P/S (penicillin-streptomycin) and IL-2 for approximately 11 days, followed by addition of cells expressing the target antigen, GD2HT1080 cells (HT1080 GD2), or K562 cells (K562 GD2), or the neuroblastoma cell line SH-SY5Y, which naturally expresses GD2.

[0039] Fig. 3A shows analytical results for the expression of EGFP from the first expression cassette measured after 24h of co-cultivation of the primary T-cells containing one of these vectors either without any additional target antigen-bearing cell line (w/o stimulation, left col.), with HT1080 GD2-bearing cells (middle col.), or with SH-SY5Y cells (right col.). On the example of eGFP as a reporter effector, these results show that the effector of the first expression cassette is essentially only expressed by the primary T-cells in the presence of cells bearing the target antigen (GD2) for the CAR, whereas in the absence of cells bearing the target antigen, essentially no expression of the effector occurs from the first expression cassette, clarifying the CAR-specific and tight induction of the effector molecule

[0040] Fig. 3B from left to right shows analytical results for the expression of human IL-12 from the first expression cassette measured after 24h of co-cultivation of the primary T-cells containing one of these vectors either without any additional target antigen-bearing cell lines (w/o stimulation, 1st col.), with SH-SY5Y cells (2nd col.), with HT1080 GD2

cells (3rd col.), or with K562 cells that express GD2 (4th col).

[0041] Further, these results show that in primary T-cells that were used as exemplary immune cells the CAR is expressed sufficiently to activate the first promoter of the first expression cassette in the presence of cells bearing its target antigen.

[0042] Fig. 3B further depicts the results for primary T-cells that did not contain the vector for comparison (untransduced), showing that the synthesis of the effector is caused by the presence of the vector in the immune cells and that there are no additional side effects or unspecific cytokine secretion in primary T cells.

[0043] For production of lentiviral particles containing the vector, the vector was cloned for transcription under the control of the RSV promoter or the CMV promoter. Viral particles were produced in 293T cells with the vector under the control of the CMV promoter or the RSV promoter and containing helper plasmids encoding VSVg pseudotype coat proteins.

[0044] Fig. 4A depicts the results of the determination of the titer of viral particles in HT1080 cells, showing that the vectors, from left to right,

NFATenhsyn.EGFP.PGK.CAR.wPRE,
NFATmIL2.EGFP.PGK.CAR.wPRE,
NFATenhsyn.humanIL12.PGK.CAR.wPRE (containing the IL12 encoding sequence in the first expression cassette), and
NFATmIL2.humanIL12.PGK.CAR.wPRE are produced at significantly higher titer under the control of the CMV promoter for transcription in production cells than under control of the RSV promoter.

[0045] Expression of the effector, represented by human IL-18, was tested in primary T-cells transduced with one of the vectors pCCL.PPT.NFATmIL2.humanIL18.PGK.CAR.PRE containing a human IL-18 encoding sequence under the control of the promoter NF A TmIL2 of SEQ ID NO: 2, pCCL.PPT.NFATenhsyn.humanIL18.PGK.CAR.PRE (SEQ ID NO: 18) containing the human IL-18 encoding sequence under the control of the promoter NFATenhsyn of SEQ ID NO: 1, wherein the CAR was a GD2-specific CAR, and in T-cells that were not genetically manipulated (untransduced, control).

[0046] Stimulation was done by co-incubation of the genetically manipulated primary T-cells or non-transduced primary T-cells as control in a 10:1 effector to target ratio of primary T-cells with target cells expressing the target antigen GD2, namely HT1080 cells expressing GD2 (HT1080 GD3/GD2, Fig. 4B, left col.) or SH-SY5Y cells (Fig. 4B, center col.), or without stimulation (w/o stimulation, Fig. 4B, right col.). These results show that in comparison to control cells (untransduced), essentially only cells containing a vector according to the invention effectively express IL-18, and that the NFATenhsyn promoter generates a significantly higher expression of the effector from the first expression cassette than the promoter NFATmIL2.

[0047] Fig. 4C depicts the analytical results of lactate dehydrogenase measurements (LDH) as an indicator for cytotoxicity of primary T-cells that were lentivirally transduced to contain a vector of the invention after co-cultivation with HT1080 cells that do not express GD2 (HT1080, negative control, left columns), or with GD2-expressing HT1080 cells (HT1080 GD3/GD2, right columns) in a target-effector ratio of 10:1 (primary T-cells to HT1080 or HT1080 GD3/GD2 cells). As a control, primary T-cells without genetic manipulation (untransduced) were used. The results show that in the presence of cells without target antigen, and especially in the presence of cells bearing the target antigen, the immune cells containing a vector according to the invention exhibit a significantly higher cytotoxicity over primary T-cells without the vector (untransduced).

Example 2: Regulatory elements of gamma retroviral vector

[0048] For embodiments, regulatory elements of the vector, especially the second promoter and the arrangement of the first and second expression cassettes in relation to one another were tested in the embodiment of a gamma retroviral vector.

[0049] As an embodiment there was used the arrangement of the first expression cassette and of the second expression cassette such that the first and the second expression cassettes are arranged on opposite strands of a double stranded nucleic acid construct with their 5'-ends adjacent one another and their 3'-ends distant from one another (antisense orientation), and the expression cassettes are between two SIN LTR (SIN). The first expression cassette from 5' to 3' consists of the NFATmIL2 representing the first promoter, dscGFP (destabilized copGFP) as a representative of the effector. The second expression cassette from 5' to 3' consists of the PGK promoter, the dTomato as a reporter representing the CAR, and a WPRE. This embodiment is schematically shown in Figure 5A.

[0050] Fig. 5C shows the generally preferred arrangement of the first expression cassette in 5' of the second expression cassette, wherein, further preferred, the first expression cassette is arranged directly adjacent to the second expression cassette, wherein both expression cassettes are arranged on one nucleic acid strand, so that both the expression cassettes are arranged in the same direction of transcription (sense orientation). A packaging signal (Ψ element) is

arranged between the 5' SIN LTR and the first promoter of the first expression cassette.

[0051] Fig. 5E shows the structure of an embodiment of the vector, in which the second expression cassette as the second promoter contains the EFS promoter to control expression of the CAR encoding sequence, in 3' to which a WPRE is adjacent.

[0052] The γ-retroviral vectors were transduced into primary T-cells, which after cultivation were stimulated with phosphate buffered saline (PBS, negative control), T-cell activating antibodies anti-CD3 and anti-CD28 (αCD3 + αCD28), or with a non-specific antibody (irrelevant antibody, αID (anti-idiotype antibody).

[0053] Figures 5B and 5D, 5F show FACS (fluorescence activated flow cytometric cell sorting) results of the expression of dsGFP from the first expression cassette after stimulation and of dTomato representing the CAR from the second expression cassette as indicated. Figures 5B and 5D show that the arrangement of the first expression cassette and adjacent thereto in 3' the second expression cassette, both on the same nucleic acid strand (Fig. 5C), upon effective stimulation by anti-CD3 and anti-CD28 results in a higher expression from both the first and the second expression cassettes than arrangement of the first expression cassette in antisense orientation and the second expression cassette on opposite strands of a double-stranded nucleic acid construct, especially with the direction of transcription pointing away from one another.

[0054] Further, Figures 5B and 5D show that mock stimulation by PBS or stimulation with an irrelevant anti-idiotype antibody (αID) results in no dscGFP expression. This shows that the nucleic acid constructs are set to specifically express the effector from the first expression cassette only upon activation of the immune cell by presence of the target antigen of the CAR. Further, these results show that the inducible promoter as the first promoter is only active upon specific activation of the immune cell.

[0055] Fig. 5F shows FACS results for primary T-cells transduced with the vector of Fig. 5E. Here, the dTomato-WPRE construct (Fig.5 C) was exchanged by a CAR expression cassette. The CAR (CAR of interest, anti-CEA (carcinoembryonic antigen)-CAR) was detected by a labelled specific antibody against the Hinge-Region of the CAR (anti-F(ab)$_2$ αIgG1-PE antibody). Fig. 5F indicates that T cells transduced with the vector displayed no dscGFP expression when stimulated with PBS, but responded with induction of dscGFP after culture with activating αCD3 antibody and αCD28 antibody. Moreover, transduced T cells induced dscGFP expression after binding to CAR-crosslinking αID antibody, providing proof that CAR-mediated signaling activates the NFAT-responsive promoter in the vector, indicating that the vector is also suitable for γ-retroviral vectors and for the EFS promoter.

Example 3: Regulatory elements of alpha retroviral vector

[0056] Regulatory elements of the vector, especially the first promoter and the arrangement of the first and second expression cassettes in relation to one another were tested in the embodiment of an alpha retroviral vector.

[0057] Primary T-cells were transduced with viral particles containing one of the exemplary vectors transferred to the alpha retroviral vector, followed by co-cultivation with GD2-expressing K562 cells for 24h (Fig 6A).

[0058] The vector containing the coding sequence for eGFP as a representative of the effector under the control of the NFATenhsyn promoter was designated pCAS.NFATenhsyn.EGFP.PGK.GD2CAR.wPRE SIN, and expression from this vector was analysed by FACS, using an anti-GD2CAR-PE antibody for detection of the CAR. Fig. 6A presents the FACS results without stimulation (w/o stimulation), and with stimulation (K562 GD3/GD2), showing that without stimulation and also in the presence of stimulation, the CAR is expressed, and that only in the presence of the target antigen GD2 for stimulation the effector, represented by eGFP (EGFP), is upregulated.

Example 4: Embodiments of the vector (first expression cassette)

[0059] The following exemplary vectors show that the vector is suitable for introduction into other immune cells besides T cells, e.g. NK92 (Fig. 6 C), primary NK and NK T cells and macrophages. The modular structure of the vector allows an adjustment to the specific properties and signaling pathways of the immune cells. An improvement of the first expression cassette can be obtained e.g. by the exchange of the promoter controlling the coding sequence of the effector module. The exchange of the NFAT promoter allele for the *nuclear factor kappa-light-chain-enhancer of activated B-cells* (NFκBenhsyn) promoter element (SEQ ID NO: 3) was found to result in an increased CAR-mediated induction of the effector, e.g. represented by eGFP, in NK92 cells (Fig. 6B and C) and primary NK cells.

[0060] Fig. 6B schematically shows exemplary vector constructs that on one nucleic acid strand between a 5' LTR and a 3' SIN LTR from 5' to 3' contain a first expression cassette consisting of a first promoter, which has SEQ ID NO: 3 (NFκBenhsyn) or nucleotides 4178 .. 4281 of SEQ ID NO: 32 (NFκBmII,2) each including a TATA box, and a coding sequence for the effector which is represented by eGFP (EGFP) or a cytokine (Cytokine), and a second expression cassette consisting of a constitutive second promoter, herein the PGK promoter having the nucleotide sequence of nucleotides No. 5158..5673 of SEQ ID NO: 8, a coding sequence for a CAR of interest and the WPRE having SEQ ID

NO: 15 (wPRE).

[0061] Fig. 6C shows the comparison between the different tested promoter elements (respectively NFATenhsyn and NFκBenhsyn) in the first expression cassette in alpha retrovirally transduced NK92 cells (pCAS.NFATenhsyn.EGFP.PGK.CAR.PRE SIN (SEQ ID NO: 26), wherein the CAR was a GD2-specific CAR, or pCAS.NFκBenhsyn.EGFP.PGK. CAR.PRE SIN (SEQ ID NO: 28). The FACS results were depicted for pCAS.NFATenhsyn.EGFP.PGK. CAR.PRE SIN in the two left graphs, and for pCAS.NFκBenhsyn.EGFP.PGK.CAR.PRE SIN in the two right graphs. The results show that the CAR is expressed from all the vector embodiments tested, and that in the absence of stimulation (w/o stimulation) essentially no effector (EGFP) is produced, whereas in the presence of the stimulating target antigen, represented by the GD2-expressing SH-SY5Y neuroblastoma cells, the representative effector eGFP is produced. This shows that the NFκBenhsyn promoter as the first promoter results in a stronger and more prominent expression of the effector encoded by the first expression cassette. The exchange of the NFAT-inducible element to the NFκB-inducible element resulted in a better and prominent upregulation of eGFP in NK92 cells indicating the adapted and improved signal transduction in these cells due to the vector according to the invention. A lentiviral vector containing the expression cassettes NFκBenhsyn.EGFP.PGK.CAR is shown in SEQ ID NO: 24.

[0062] Example 5: Embodiments of the vector expressing hIL21 or hTRAILExemplary embodiments of the vector, which in the first expression cassette encode human interleukin 21 (hIL21) or human tumor necrosis factor-related apoptosis-inducing ligand (hTRAIL) under the control of a CAR inducible promoter, with a second expression cassette arranged in 3' and in sense orientation and directly adjacent to the first expression cassette. The second expression cassette encodes a CAR having a binding domain that is specific for the G_{D2} tumor antigen of glioblastoma cells. As generally preferred, also these nucleic acid constructs are devoid of a poly-adenylation signal between the first expression cassette and the second expression cassette. Fig. 7A schematically shows the arrangement of elements of the nucleic acid constructs from 5' to 3', wherein the coding sequence for the effector molecule hTRAII, is depicted beneath the coding sequence for hIL21, schematically indicating that the hTRAII, encoding sequence is inserted in the same place as the hIL21 encoding sequence between the first promoter and the second expression cassette. These nucleic acid constructs contain flanking LTRs, a 5'SIN LTR and a 3'SIN LTR. The second expression cassette contains the PGK promoter, which controls constitutive expression of the G_{D2}-specific CAR (G_{D2}CAR). The nucleic acid sequence of the lentiviral vector encoding hIL21 is termed pCCL.PPT.NFATenhsyn.humanIL21co.PGK.CAR.PRE, the nucleic acid sequence is given in SEQ ID NO: 34, the nucleic acid sequence of the lentiviral vector encoding hTRAII, is termed pCCL.PPT.NFATenhsyn.TRAIL.PGK.CAR.PRE, the nucleic acid sequence is given in SEQ ID NO: 35.

[0063] Human primary T-cells were transduced separately with one of the vectors shown in Fig. 7A, and the transduced T-cells were co-cultivated with or without G_{D2} expressing human glioblastoma cells that were obtained from a patient and cultivated. After two days of co-cultivation of the glioblastoma cells with the T-cells that were transduced with the vector encoding hIL21, or of cultivation of the transduced T-cells alone, IL21 was determined by ELISA. In contrast to T-cells cultivated without glioblastoma cells or T-cells transduced with a vector that was devoid of the G_{D2}-CAR but contained only the first expression cassette encoding hIl,21 cultivated in presence of or in absence of glioblastoma cells that were used as negative controls, the T-cells containing the vector according to the invention express hIL21 only in presence of glioblastoma cells. The results are shown in Fig. 7B, wherein n.d. denotes that hIL21 was not detectable in the controls, indicating that hIL21 was below detection levels.

[0064] For the T-cells that were transduced with the vector encoding hTRAII, as the effector molecule, intracellular TRAIL was analysed from collected cells by ELISA after 2 days, 6 days and after 9 days of co-cultivation with glioblastoma cells. As negative controls, the untransduced (Mock) T-cells alone and T-cells only (i.e. without glioblastoma coculture) transduced with the vector according to the invention with both the first (hTRAIL) and the second (G_{D2}-CAR) expression cassette were cultured. The ELISA results of cell lysates show that only the T-cells transduced with the vector according to the invention with both the first and the second expression cassettes produced hTRAIL, and only in presence of the glioblastoma cells. Expression of the exemplary effector molecule hTRAII, increased significantly over the time of the cultivation, namely at day 2 10pg/mL, at day 6 84.7 pg/mL, at day 9212.8 pg/mL. Results are shown in Fig. 7C, wherein n.d. denotes that hTRAIL was not detectable in the controls, indicating that hTRAII, was below detection levels.

Example 6: Immune cells containing the vector

[0065] Using EGFP as a representative for an effector molecule under the control of the first promoter and a second expression cassette expressing a G_{D2}-CAR in a nucleic acid construct according to the invention, NK-92 cells were transduced and co-cultivated with patient-derived glioblastoma cells expressing the target antigen G_{D2}, and as a control without these stimulating cells. In one embodiment, the vector NFATsyn.EGFP-GD2CAR as the first promoter contained the Nasty promoter, in another embodiment, the vector NFATmIL2.EGFP-GD2CAR contained the NFATmIL2 promoter, and in a further embodiment the vector NFκBsyn.EGFP-GD2CAR contained the NFκBsyn promoter as the first promoter. These constructs contained the PGK promoter as the second promoter to constitutively express the CAR, and in 3' to the second expression cassette, which was arranged sense orientation in 3' to the first expression cassette, contained

a wPRE and in 5' to the first expression cassette contained a packaging signal, an RRE and a PPT, the construct flanked by SIN LTRs, and except for the different first promoters, these nucleic acid constructs were identical.

[0066] The results are shown in Fig. 8, wherein Fig. 8A shows a bar graph of a summary of flow cytometric analysis of unsorted NFATsyn.EGFP-GD2CAR- and NFATmIL2.EGFP-GD2CAR-modified (MOI 10) NK-92 cells after a 24 h-lasting co-culture with different target cells. GD2CAR-positive NK-92 cells were detected via antibody staining of scFv-CAR-region. Co-cultures were performed in a 10:1 effector:target (E:T; NK-92 cells : glioblastoma cells) ratio. Only a slight NFAT-driven EGFP expression after antigen recognition was detectable; (mean values ± SD; n=3, biological replicates).

[0067] Fig. 8B shows a bar graph of a summary of flow cytometric analysis of EGFP upregulation in unsorted NFκB-syn.EGFP-GD2CAR-positive (MOI 10) NK-92 cells after co-culture with different target cells in a 10:1 effector:target (E:T) ratio for 24 h. GD2CAR-positive NK-92 cells were identified via antibody staining of scFv-CAR-region. A prominent EGFP expression was detected dependent on GD2-recognition of target cells in co-culture; (mean values ± SD; n=5). In Fig. 8A and 8B, the columns from left to right are without (w/o) stimulation, with stimulation by HT1080 (HT1080, negative comparison), with stimulation by HT1080 expressing GD2 (HT1080 GD2, positive), and stimulation by SH-SY5Y.

[0068] Fig. 8C shows a bar graph of a summary of flow cytometric analyses of a co-culture with modified primary NK cells and GD2-positive and GD2-negative suspension target cells after 24 h. Transduced (multiplicity of infection (MOI) 10) and unsorted primary NK cells of three different donors were co-cultivated with target cells in a 10:1 target effector (T:E) ratio. GD2CAR-positive primary NK cells were identified via antibody staining against CD56 and against the single chain variable fragment (scFv)-CAR-region. As a negative comparison KG-1α was used, for stimulation KG1α expressing GD2 (KG1α GD2). A clear inducible NFκB-driven EGFP expression was detected after antigen recognition.

[0069] Fig. 8D shows exemplary flow cytometric analyses (FACS) of a 24 h-lasting co-culture of sorted modified NK-92 cells with and without patient-derived primary glioblastoma cells. A specific EGFP upregulation was seen after tumor recognition. The bar graph of Fig. 8E shows a comparison of specific NFκB-driven EGFP upregulation after 24 h and 48 h.

[0070] These results show that immune cells containing the vector in the presence of cells that carry the target antigen of the CAR specifically produce the effector molecule that is encoded by the first expression cassette. Further, these results show that in immune cells containing the vector, the NFκBsyn promoter as the first promoter can be contained to generate a stronger expression of the effector molecule, and the NFATenhsyn promoter as the first promoter can be contained to generate a weaker expression of the effector molecule. Furthermore, the results show that the choice of the promoter in the first expression cassette can improve the expression of the inducible gene of interest and is dependent on the immune cell.

Claims

1. Nucleic acid construct for expression of an effector molecule in response to the presence of a target antigen of a chimeric antigen receptor (CAR) or a T-cell receptor (TCR), **characterized in that** the nucleic acid construct in one strand comprises a first expression cassette, comprising a first promoter which is inducible by binding of a CAR or TCR to its target antigen to control expression of a coding sequence for the effector molecule, and a second expression cassette encoding the CAR or TCR under the control of a constitutive second promoter, wherein the nucleic acid construct is part of a lentiviral vector, of an alpha (α)-retroviral vector or of a gamma (γ)-retroviral vector, and wherein the first expression cassette is arranged in 5' of the second expression cassette, wherein between the first expression cassette and the second expression cassette there is no poly-adenylation signal and wherein a poly-adenylation signal is contained in an LTR that is arranged in 3' of the second expression cassette.

2. Nucleic acid construct according to one of the preceding claims, **characterized in that** the first promoter is selected from SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, and SEQ ID NO: 31.

3. Nucleic acid construct according to one of the preceding claims, **characterized in that** the constitutive second promoter is selected from a PGK promoter having the nucleotide sequence of nucleotides No. 5158..5673 of SEQ ID NO: 8, an EF-1 alpha promoter having SEQ ID NO: 11, and an SFFV promoter having SEQ ID NO: 10.

4. Nucleic acid construct according to one of the preceding claims, **characterized in that** a WPRE is arranged at the 3' end of the CAR encoding sequence.

5. Nucleic acid construct according to one of the preceding claims, **characterized in that** the nucleic acid construct only contains a poly-adenylation signal that is contained in an LTR that is arranged in 3' of the second expression cassette.

6. Nucleic acid construct according to one of the preceding claims, **characterized in that** a viral packaging signal is arranged in 5' of the first expression cassette.

7. Nucleic acid construct according to one of the preceding claims, **characterized in that** the first expression cassette encodes the effector molecule by SEQ ID NO: 4 or by SEQ ID NO: 6.

8. Nucleic acid construct according to one of the preceding claims, wherein the arrangement of the first expression cassette and of the second expression cassette is arranged between a 5' LTR and a 3' SIN LTR.

9. Nucleic acid construct according to claim 8, **characterized in that** the LTR and SIN LTR elements are of lentiviral, of alpha-retroviral, or of gamma-retroviral origin.

10. Nucleic acid construct according to one of the preceding claims, **characterized in that** for expression in a production cell, the nucleic acid construct is under the control of a strong promoter for driving transcription of the genomic packageable viral mRNA, e.g. selected from the CMV promoter, RSV promoter or HIV and MLV-derived promoters.

11. Nucleic acid construct according to one of the preceding claims, wherein the nucleic acid construct is contained in a viral particle.

12. Nucleic acid construct according to one of the preceding claims, **characterized in that** the intracytoplasmatic effector domain of the CAR is the intracellular effector domain of DAP 12 or DAP 10 or the intracytoplasmatic effector domain of the CAR comprises the CD3ζ domain, and in each case optionally an adjacent CD28 or 4-1BB domain.

13. Immune cell, containing a nucleic acid construct according to one of the preceding claims for use in the treatment of cells expressing or presenting the target antigen of the CAR or TCR.

14. Immune cell according to claim 13 for use in the treatment of cells which are virus-infected cells or tumour cells, wherein the CAR is specific for a target antigen which is selected from viral antigens and tumour antigens, wherein the immune cell is a CD8+ (cytotoxic) T-cell, a primary NK-cell or NK T-cell, a NK92-cell, a macrophage, or a dendritic cell.

15. Immune cell according to claim 13 for use in the treatment of an autoimmune disease or for use in the induction of tolerance for a target antigen, wherein the CAR is specific for the target antigen wherein the immune cell is a regulatory CD4+CD25+FOXP3+ regulatory T cell or an alternative immunomodulatory cell type, e.g. selected from NK cells, macrophages, double negative regulatory T cell and a mesenchymal stroma cell.

16. *In vitro* process for producing an immune cell comprising the steps of providing an immune cell and introducing a nucleic acid construct according to one of claims 1 to 12 into the immune cell.

17. *In vitro* process according to claim 16, wherein the immune cell originates from a patient, the nucleic acid construct is introduced into the immune cell *in vitro,* for producing an immune cell for use in the treatment of cells expressing or presenting the target antigen of the CAR or TCR in the patient.

18. *In vitro* process for producing viral particles containing a nucleic acid construct according to one of claims 1 to 12, comprising expressing the nucleic acid construct in a mammalian cell and co-expressing the viral structural proteins and replication enzymes (gag-pol), and the retroviral envelope protein (env), and optionally a rev protein.

Patentansprüche

1. Nukleinsäurekonstrukt zur Expression eines Effektormoleküls in Reaktion auf die Anwesenheit eines Zielantigens eines chimären Antigenrezeptors (CAR) oder eines TZell-Rezeptors (TCR), **dadurch gekennzeichnet, dass** das Nukleinsäurekonstrukt in einem Strang eine erste Expressionskassette umfasst, die einen ersten Promotor umfasst, der durch Bindung eines CAR oder TCR an sein Zielantigen induzierbar ist, um die Expression einer kodierenden Sequenz für das Effektormolekül zu kontrollieren, und eine zweite Expressionskassette, die den CAR oder TCR unter der Kontrolle eines konstitutiven zweiten Promotors kodiert, wobei das Nukleinsäurekonstrukt Teil eines lentiviralen Vektors, eines alpha (α)-retroviralen Vektors oder eines gamma (γ)-retroviralen Vektors ist, und wobei die erste Expressionskassette in 5' der zweiten Expressionskassette angeordnet ist, wobei zwischen der ersten Ex-

pressionskassette und der zweiten Expressionskassette kein Polyadenylierungssignal vorhanden ist und wobei ein Polyadenylierungssignal in einem LTR enthalten ist, das in 3' der zweiten Expressionskassette angeordnet ist.

2. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** der erste Promotor ausgewählt ist aus SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 und SEQ ID NO: 31.

3. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** der konstitutive zweite Promotor unter einem PGK-Promotor mit der Nukleotidsequenz der Nukleotide Nr. 5158...5673 von SEQ ID NO: 8, einem EF-1 alpha-Promotor mit SEQ ID NO: 11 und einem SFFVPromotor mit SEQ ID NO: 10 ausgewählt ist.

4. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** am 3'-Ende der CAR-kodierenden Sequenz ein WPRE angeordnet ist.

5. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** das Nukleinsäurekonstrukt nur ein Polyadenylierungssignal enthält, das in einem LTR enthalten ist, das in 3' der zweiten Expressionskassette angeordnet ist.

6. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** in 5' der ersten Expressionskassette ein virales Verpackungssignal angeordnet ist.

7. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die erste Expressionskassette das Effektormolekül durch SEQ ID NO: 4 oder durch SEQ ID NO: 6 kodiert.

8. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, wobei die Anordnung der ersten Expressionskassette und der zweiten Expressionskassette zwischen einem 5' LTR und einem 3' SIN LTR angeordnet ist.

9. Nukleinsäurekonstrukt nach Anspruch 8, **dadurch gekennzeichnet, dass** die LTR und SIN-LTR-Elemente lentiviralen, alpha-retroviralen oder gamma-retroviralen Ursprungs sind.

10. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** das Nukleinsäurekonstrukt zur Expression in einer Produktionszelle unter der Kontrolle eines starken Promotors steht, um die Transkription der genomisch verpackbaren viralen mRNA zu steuern, z. B. ausgewählt unter dem CMV-Promotor, dem RSV-Promotor oder von HIV und MLV abgeleiteten Promotoren.

11. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, wobei das Nukleinsäurekonstrukt in einem viralen Partikel enthalten ist.

12. Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche, **dadurch gekennzeichnet, dass** die intrazytoplasmatische Effektor-Domäne des CAR die intrazelluläre Effektor-Domäne von DAP12 oder DAP10 ist oder die intrazytoplasmatische Effektor-Domäne des CAR die CD3ζ-Domäne und in jedem Fall optional eine angrenzende CD28- oder 4-1BB-Domäne umfasst.

13. Immunzelle, die ein Nukleinsäurekonstrukt nach einem der vorhergehenden Ansprüche enthält, zur Verwendung bei der Behandlung von Zellen, die das Zielantigen des CAR oder TCR exprimieren oder präsentieren.

14. Immunzelle nach Anspruch 13 zur Verwendung bei der Behandlung von Zellen, die virusinfizierte Zellen oder Tumorzellen sind, wobei der CAR spezifisch für ein Zielantigen ist, das aus viralen Antigenen und Tumorantigenen ausgewählt ist, wobei die Immunzelle eine CD8+ (zytotoxische) T-Zelle, eine primäre NK-Zelle oder NK-T-Zelle, eine NK92-Zelle, ein Makrophage oder eine dendritische Zelle ist.

15. Immunzelle nach Anspruch 13 zur Verwendung bei der Behandlung einer Autoimmunerkrankung oder zur Verwendung bei der Induktion von Toleranz für ein Zielantigen, wobei der CAR spezifisch für das Zielantigen ist, wobei die Immunzelle eine regulatorische CD4+CD25+FOXP3+ regulatorische T-Zelle oder ein alternativer immunmodulatorischer Zelltyp ist, z.B. ausgewählt aus NK-Zellen, Makrophagen, doppelt negativen regulatorischen T-Zellen und einer mesenchymalen Stromazelle.

16. In vitro Verfahren zur Herstellung einer Immunzelle, das die Schritte des Bereitstellens einer Immunzelle und des

Einführens eines Nukleinsäurekonstrukts nach einem der Ansprüche 1 bis 12 in die Immunzelle umfasst.

17. In vitro Verfahren nach Anspruch 16, bei dem die Immunzelle von einem Patienten stammt, das Nukleinsäurekonstrukt in die Immunzelle in vitro eingeführt wird, um eine Immunzelle zur Verwendung bei der Behandlung von Zellen zu erzeugen, die das Zielantigen des CAR oder TCR im Patienten exprimieren oder präsentieren.

18. In vitro Verfahren zur Herstellung viraler Partikel, die ein Nukleinsäurekonstrukt nach einem der Ansprüche 1 bis 12 enthalten, das die Expression des Nukleinsäurekonstrukts in einer Säugetierzelle und die Koexpression der viralen Strukturproteine und Replikationsenzyme (gag-pol) und des retroviralen Hüllproteins (env) und gegebenenfalls eines rev-Proteins umfasst.

Revendications

1. Construction d'acide nucléique pour l'expression d'une molécule effectrice en réponse à la présence d'un antigène cible d'un récepteur d'antigène chimérique (CAR) ou d'un récepteur de cellule T (TCR), **caractérisée en ce que** la construction d'acide nucléique dans un brin comprend une première cassette d'expression, comprenant un premier promoteur inductible par la liaison d'un CAR ou d'un TCR à son antigène cible pour contrôler l'expression d'une séquence codante pour la molécule effectrice, et une seconde cassette d'expression codant le CAR ou le TCR sous le contrôle d'un second promoteur constitutif, dans lequel la construction d'acide nucléique fait partie d'un vecteur lentiviral, d'un vecteur alpha (α)-rétroviral ou d'un vecteur gamma (y)- rétroviral, et dans lequel la première cassette d'expression est disposée en 5' de la deuxième cassette d'expression, dans lequel entre la première cassette d'expression et la deuxième cassette d'expression il n'y a pas de signal de poly-adénylation et dans lequel un signal de poly-adénylation est contenu dans une LTR qui est disposée en 3' de la deuxième cassette d'expression.

2. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** le premier promoteur est choisi parmi SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3 et SEQ ID NO: 31.

3. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** le second promoteur constitutif est choisi parmi un promoteur PGK ayant la séquence nucléotidique des nucléotides n° 5158 à 5673 de SEQ ID NO: 8, un promoteur EF-1 alpha ayant SEQ ID NO: 11, et un promoteur SFFV ayant SEQ ID NO: 10.

4. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait qu'**un WPRE est disposé à l'extrémité 3' de la séquence codant pour le CAR.

5. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** la construction d'acide nucléique ne contient qu'un signal de poly-adénylation contenu dans une LTR disposée en 3' de la seconde cassette d'expression.

6. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait qu'**un signal d'empaquetage viral est disposé en 5' de la première cassette d'expression.

7. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** la première cassette d'expression code la molecule effectrice par SEQ ID NO: 4 ou par SEQ ID NO: 6.

8. Construction d'acide nucléique selon l'une des revendications précédentes, dans laquelle l'arrangement de la première cassette d'expression et de la deuxième cassette d'expression est disposé entre une LTR 5' et une LTR 3' SIN.

9. Construction d'acide nucléique selon la revendication 8, **caractérisée par le fait que** les éléments LTR et SIN LTR sont d'origine lentivirale, alpha-rétrovirale ou gamma-rétrovirale.

10. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** pour l'expression dans une cellule de production, la construction d'acide nucléique est sous le contrôle d'un promoteur fort pour entraîner la transcription de l'ARNm viral génomique empaquetable, par exemple, choisi parmi le promoteur du CMV, le promoteur du RSV ou les promoteurs dérivé du VIH et dérivé du MI,V.

11. Construction d'acide nucléique selon l'une des revendications précédentes, dans laquelle la construction d'acide nucléique est contenue dans une particule virale.

12. Construction d'acide nucléique selon l'une des revendications précédentes, **caractérisée par le fait que** le domaine effecteur intracytoplasmique du CAR est le domaine effecteur intracellulaire de DAP12 ou DAP10 ou que le domaine effecteur intracytoplasmique du CAR comprend le domaine CD3ζ et, dans chaque cas, éventuellement un domaine CD28 ou 4-1BB adjacent.

13. Cellule immunitaire contenant une construction d'acide nucléique selon l'une des revendications précédentes, pour utilisation dans le traitement de cellules exprimant ou présentant l'antigène cible du CAR ou du TCR.

14. Cellule immunitaire selon la revendication 13 pour utilization dans le traitement de cellules infectées par un virus ou de cellules tumorales, dans laquelle la CAR est spécifique d'un antigène cible choisi parmi les antigènes viraux et les antigènes tumoraux, dans laquelle la cellule immunitaire est une cellule T (cytotoxique) CD8+, une cellule NK primaire ou une cellule T NK, une cellule NK92, un macrophage ou une cellule dendritique.

15. Cellule immunitaire selon la revendication 13 pour utilisation dans le traitement d'une maladie auto-immune ou pour utilisation dans l'induction de la tolérance à un antigène cible, dans laquelle le CAR est spécifique de l'antigène cible, dans laquelle la cellule immunitaire est une cellule T régulatrice CD4+CD25+FOXP3+ ou un autre type de cellule immunomodulatrice, par exemple choisie parmi les cellules NK, les macrophages, les cellules T régulatrices doublement négatives et une cellule du stroma mésenchymateux.

16. Procédé in vitro de production d'une cellule immunitaire comprenant les étapes de fourniture d'une cellule immunitaire et d' introduction d'une construction d'acide nucléique selon l'une des revendications 1 à 12 dans la cellule immunitaire.

17. Procédé in vitro selon la revendication 16, dans lequel la cellule immunitaire provient d'un patient, la construction d'acide nucléique est introduite dans la cellule immunitaire in vitro, pour produire une cellule immunitaire pour utilisation dans le traitement des cellules exprimant ou présentant l'antigène cible du CAR ou du TCR dans le patient.

18. Procédé in vitro de production de particules virales contenant une construction d'acide nucléique selon l'une des revendications 1 à 12, comprenant l'expression de la construction d'acide nucléique dans une cellule de mammifère et la co-expression des protéines structurelles virales et des enzymes de réplication (gag-pol), et de la protéine d'enveloppe rétrovirale (env), et éventuellement d'une protéine rev.

Fig. 1

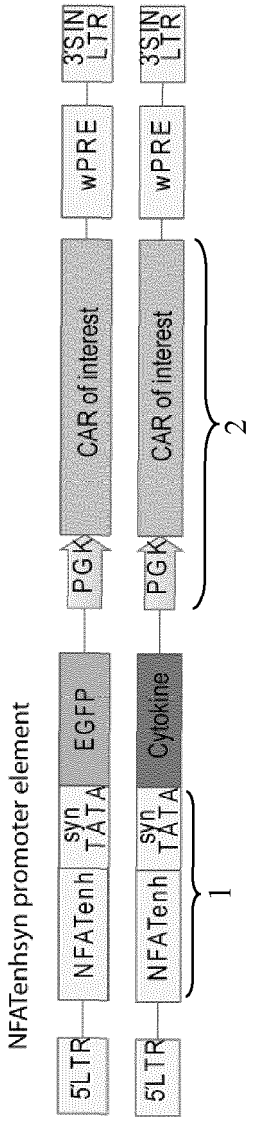


Fig. 2

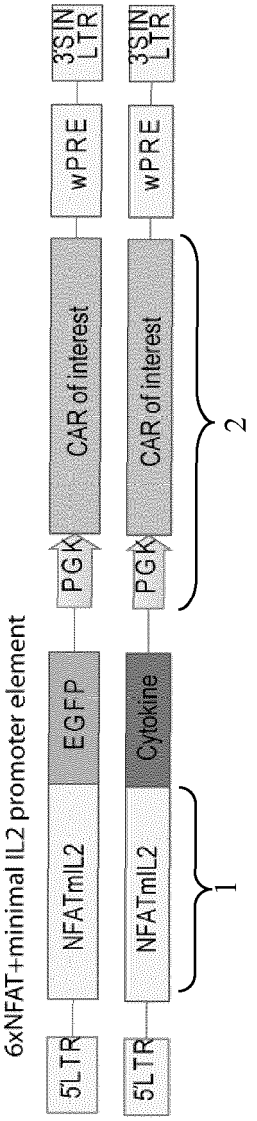


Fig. 3

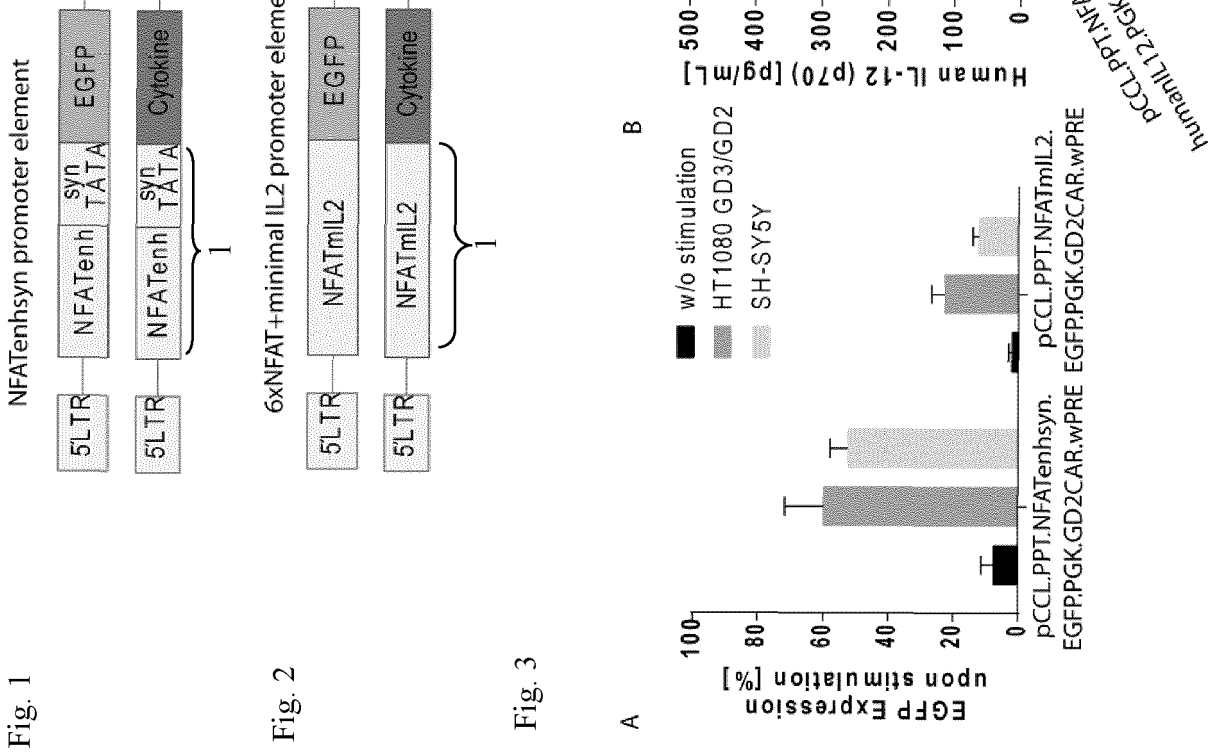


Fig. 4

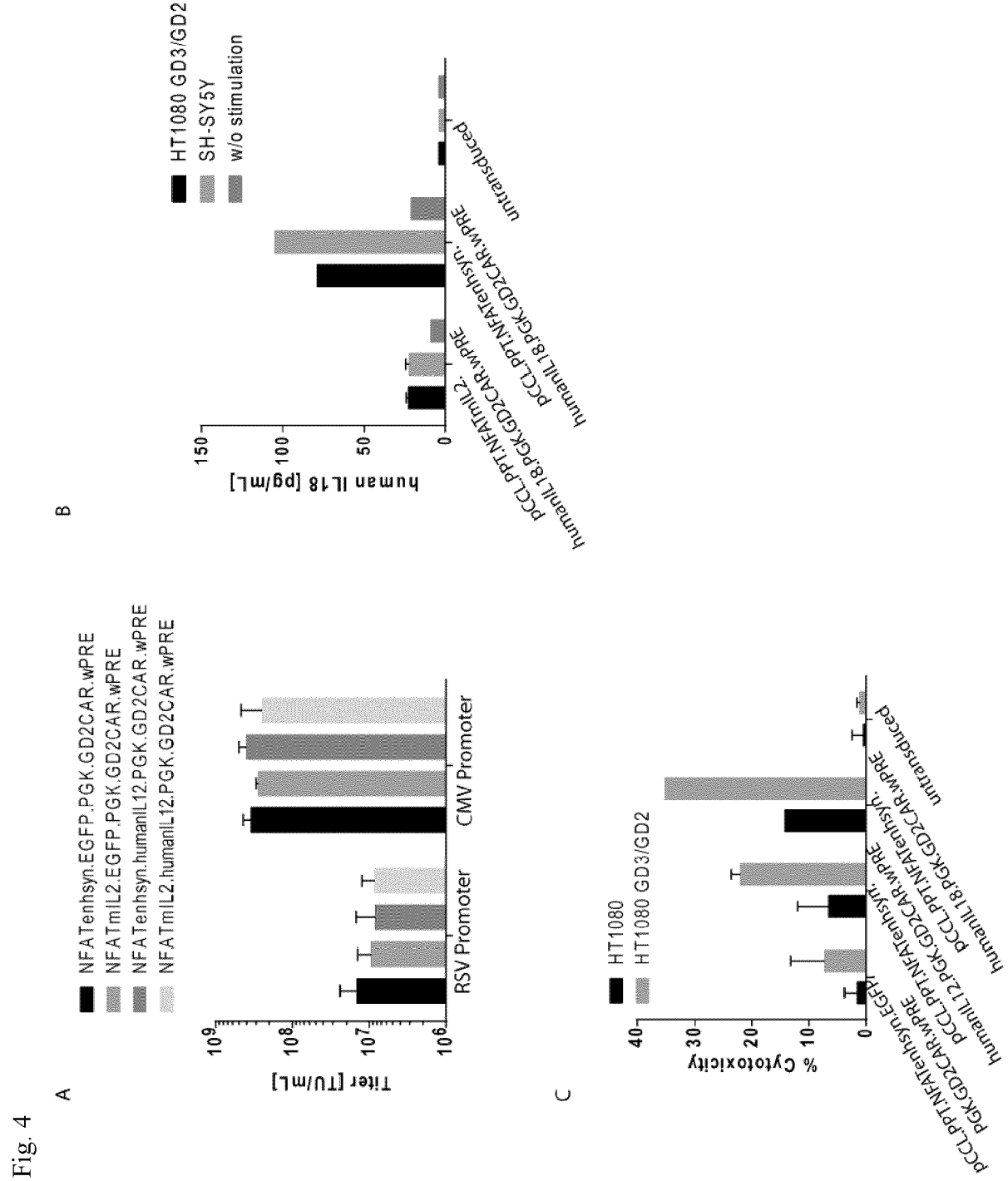

A
NFATenhsyn.EGFP.PGK.GD2CAR.wPRE
NFATmIL2.EGFP.PGK.GD2CAR.wPRE
NFATenhsyn.humanIL12.PGK.GD2CAR.wPRE
NFATmIL2.humanIL12.PGK.GD2CAR.wPRE
Titer [TU/mL]
RSV Promoter
CMV Promoter
B
HT1080 GD3/GD2
SH-SY5Y
w/o stimulation
human IL18 [pg/mL]
pCCL.pPT.NFATmIL2.
humanIL18.pGK.GD2CAR.wPRE
pCCL.pPT.NFATenhsyn.
humanIL18.pGK.GD2CAR.wPRE
untransduced
C
HT1080
HT1080 GD3/GD2
% Cytotoxicity
pCCL.pPT.NFATenhsyn.EGFP.
pGK.GD2CAR.wPRE
pCCL.pPT.NFATenhsyn.
humanIL12.pGK.GD2CAR.wPRE
untransduced

Fig. 5

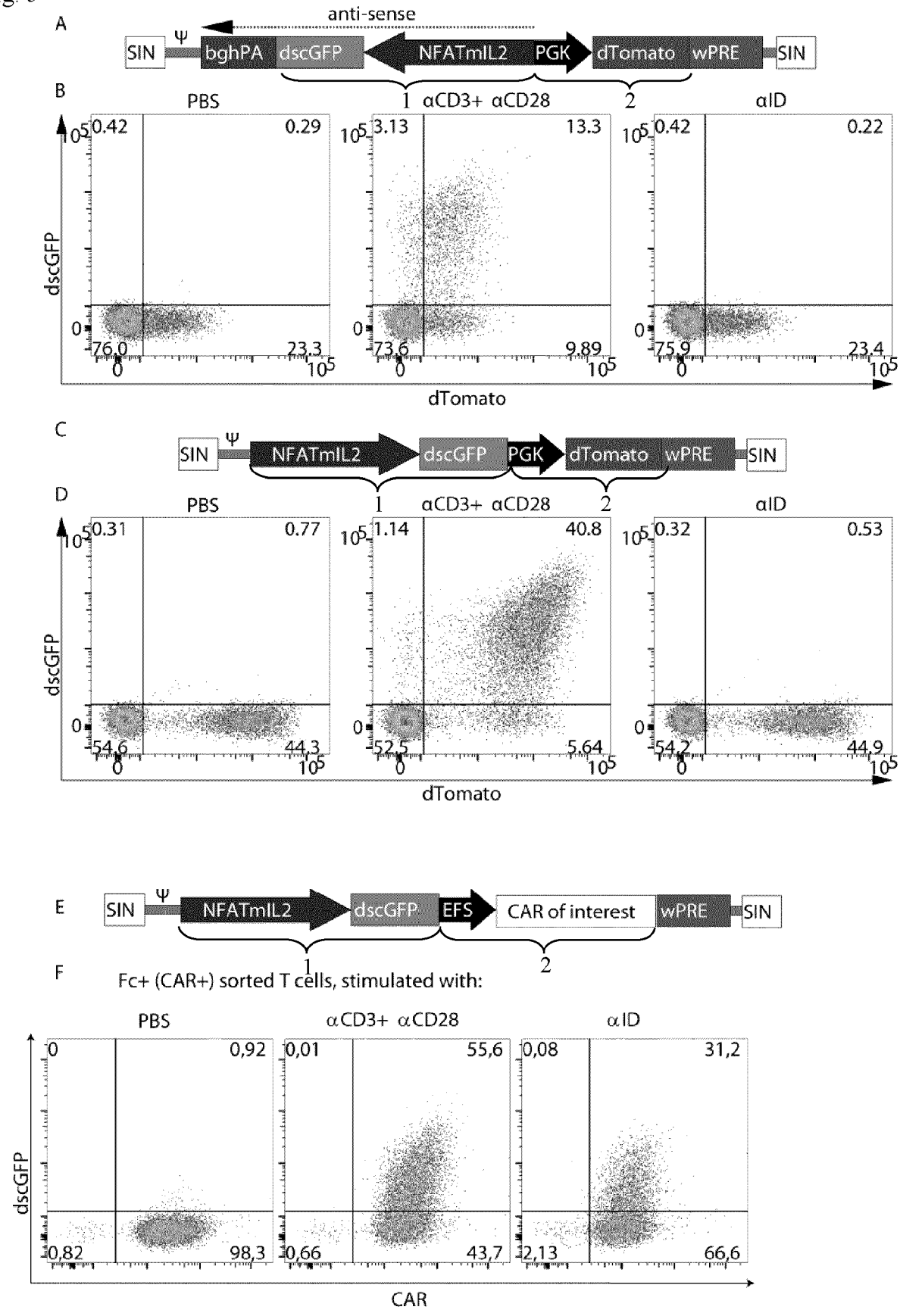

A
anti-sense
SIN
Ψ
bghPA
dscGFP
NFATmIL2
PGK
dTomato
wPRE
SIN
1
2
B
PBS
αCD3+ αCD28
αID
0.42
0.29
3.13
13.3
0.42
0.22
76.0
23.3
73.6
9.89
75.9
23.4
dscGFP
dTomato
C
SIN
Ψ
NFATmIL2
dscGFP
PGK
dTomato
wPRE
SIN
1
2
D
PBS
αCD3+ αCD28
αID
0.31
0.77
1.14
40.8
0.32
0.53
54.6
44.3
52.5
5.64
54.2
44.9
dscGFP
dTomato
E
SIN
Ψ
NFATmIL2
dscGFP
EFS
CAR of interest
wPRE
SIN
1
2
F
Fc+ (CAR+) sorted T cells, stimulated with:
PBS
αCD3+ αCD28
αID
0
0,92
0,01
55,6
0,08
31,2
0,82
98,3
0,66
43,7
2,13
66,6
dscGFP
CAR

Fig. 6

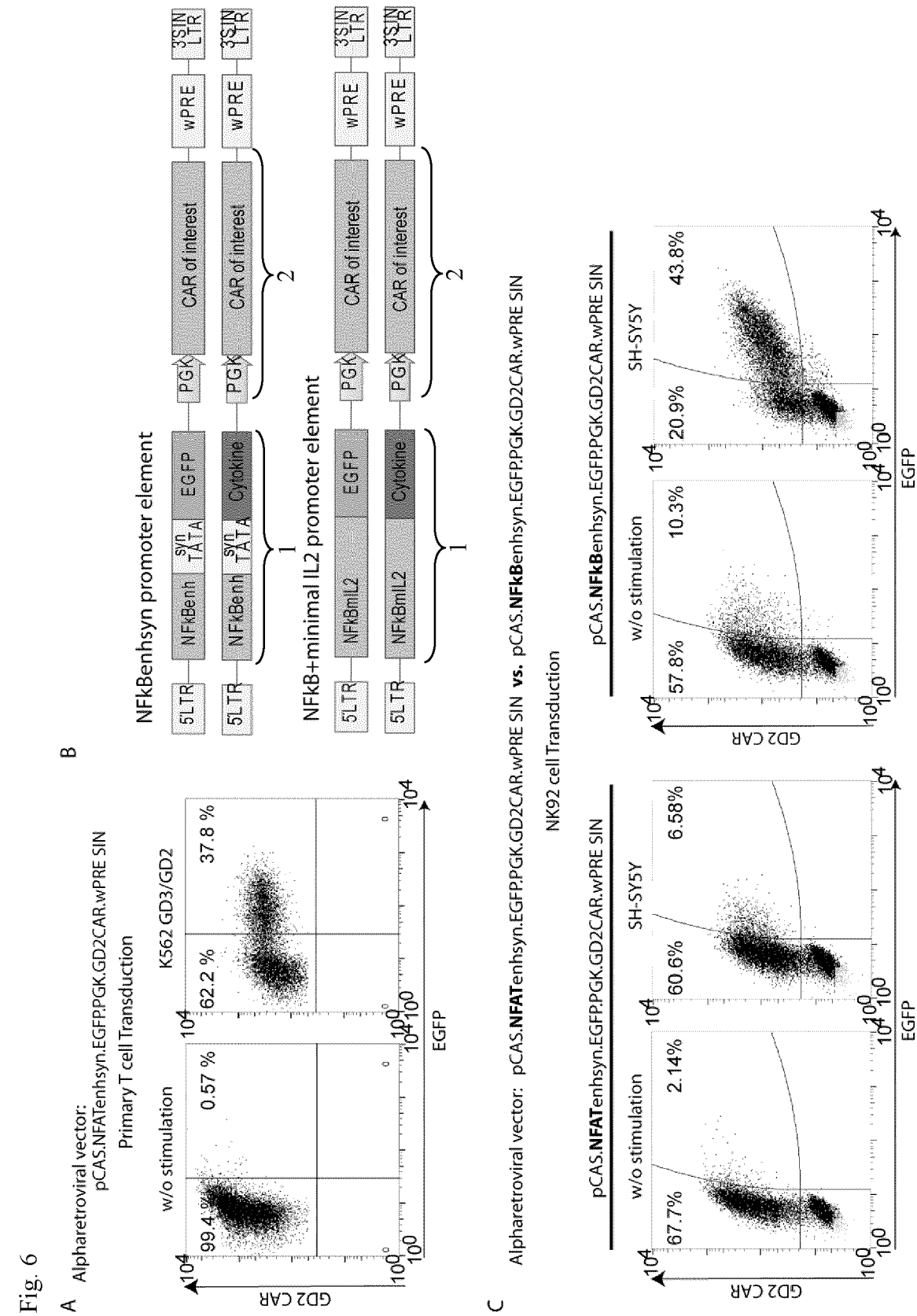

A
Alpharetroviral vector:
pCAS.NFATenhsyn.EGFP.PGK.GD2CAR.wPRE SIN
Primary T cell Transduction
w/o stimulation
K562 GD3/GD2
99.4 %
0.57 %
62.2 %
37.8 %
GD2 CAR
EGFP
B
NFkBenhsyn promoter element
5LTR
NFkBenh
syn TATA
EGFP
PGK
CAR of interest
wPRE
3SIN LTR
5LTR
NFkBenh
syn TATA
Cytokine
PGK
CAR of interest
wPRE
3SIN LTR
1
2
NFkB+minimal IL2 promoter element
5LTR
NFkBmIL2
EGFP
PGK
CAR of interest
wPRE
3SIN LTR
5LTR
NFkBmIL2
Cytokine
PGK
CAR of interest
wPRE
3SIN LTR
1
2
C
Alpharetroviral vector: pCAS.NFATenhsyn.EGFP.PGK.GD2CAR.wPRE SIN vs. pCAS.NFkBenhsyn.EGFP.PGK.GD2CAR.wPRE SIN
NK92 cell Transduction
pCAS.NFATenhsyn.EGFP.PGK.GD2CAR.wPRE SIN
w/o stimulation
SH-SY5Y
67.7%
2.14%
60.6%
6.58%
pCAS.NFkBenhsyn.EGFP.PGK.GD2CAR.wPRE SIN
w/o stimulation
SH-SY5Y
57.8%
10.3%
20.9%
43.8%
GD2 CAR
EGFP

Fig. 7

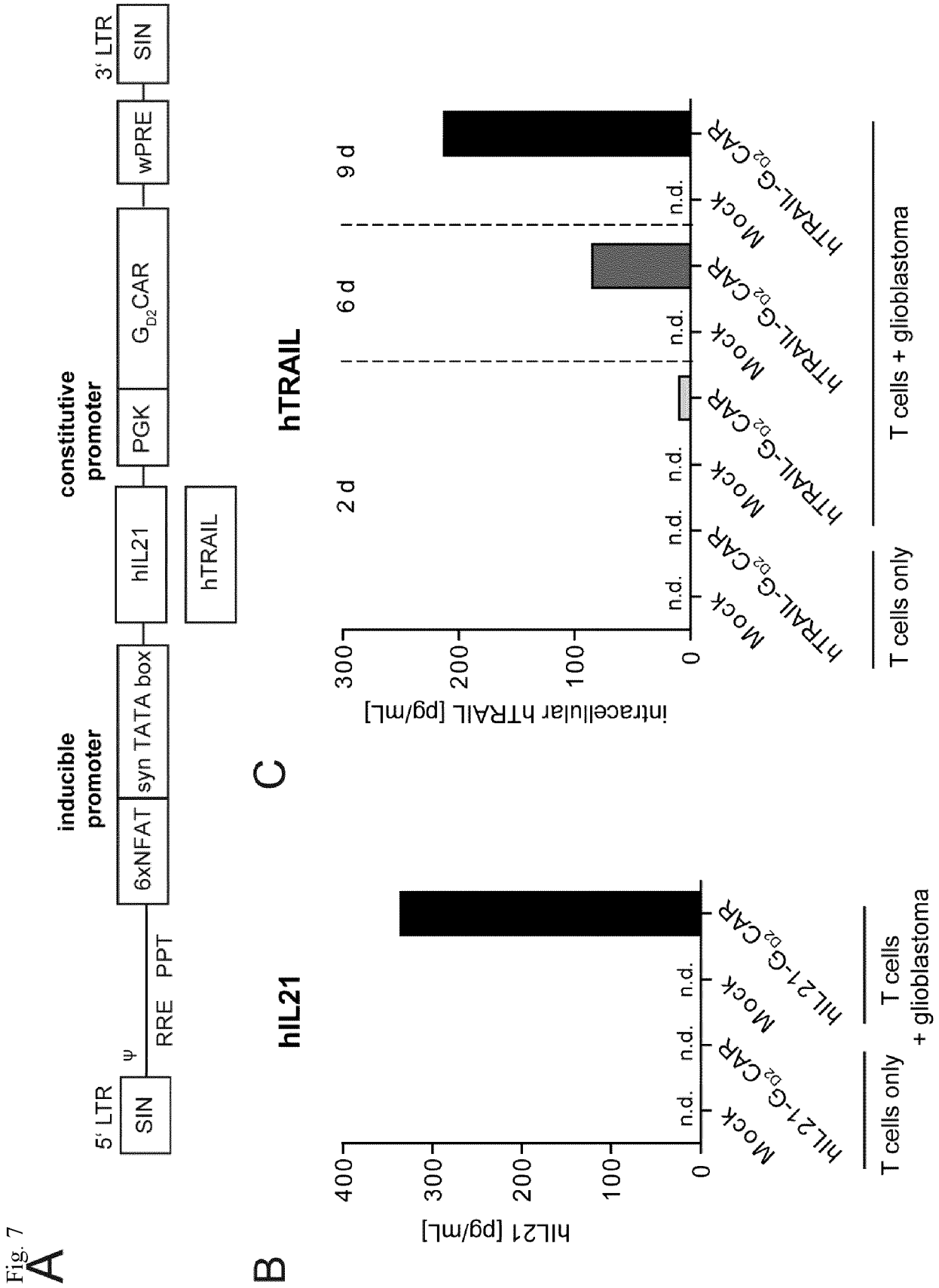
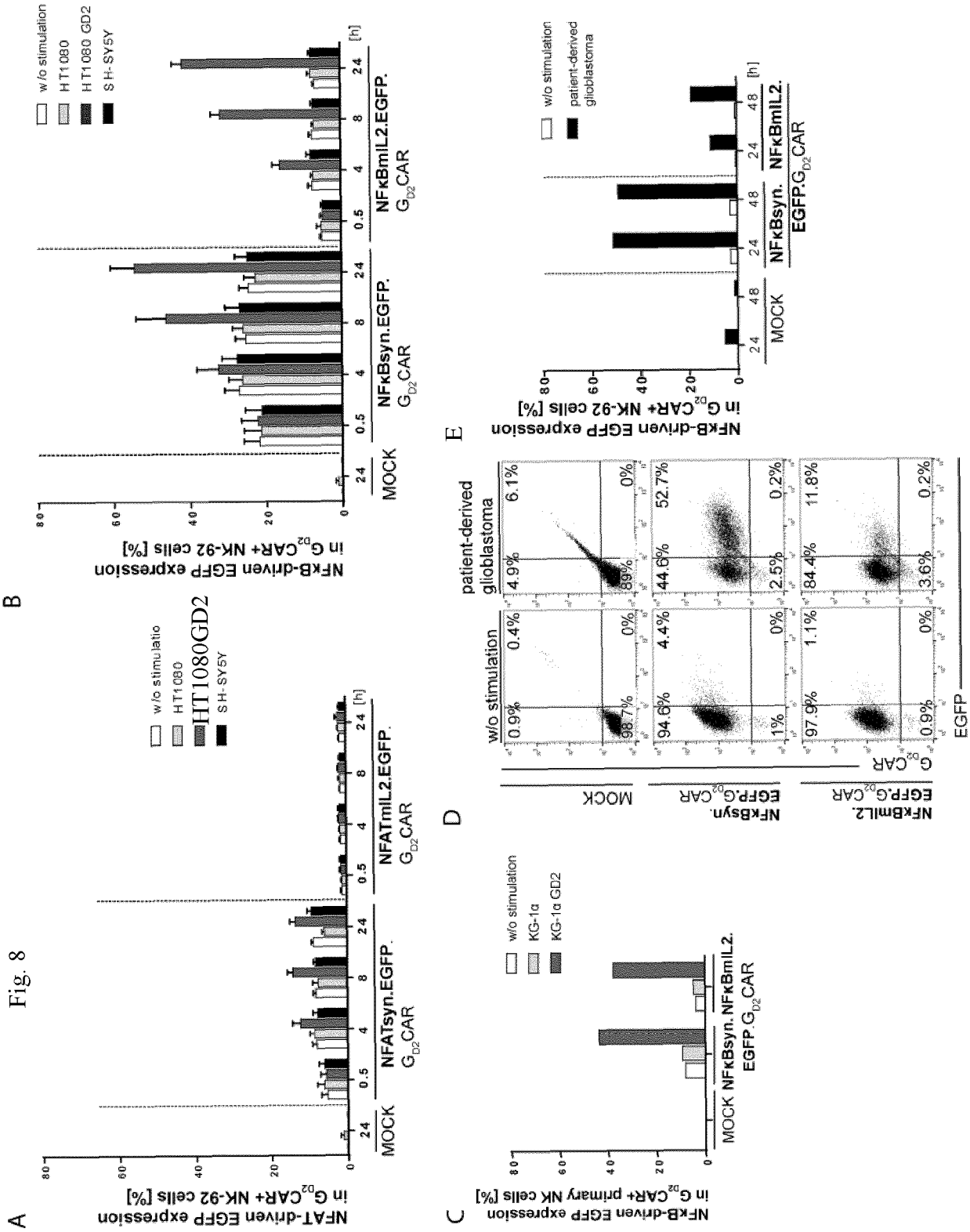

A
5' LTR
SIN
ψ
RRE
PPT
inducible promoter
6xNFAT
syn TATA box
hIL21
hTRAIL
constitutive promoter
PGK
G_{D2}CAR
wPRE
3' LTR
SIN
B
hIL21
hIL21 [pg/mL]
400
300
200
100
0
n.d.
Mock
hIL21-G_{D2}CAR
T cells only
T cells + glioblastoma
C
hTRAIL
intracellular hTRAIL [pg/mL]
300
200
100
0
2 d
6 d
9 d
Mock
hTRAIL-G_{D2}CAR
T cells only
T cells + glioblastoma

Fig. 8


A
B
C
D
E
w/o stimulation
HT1080
HT1080GD2
SH-SY5Y
NFAT-driven EGFP expression in G$_{D2}$CAR+ NK-92 cells [%]
NFκB-driven EGFP expression in G$_{D2}$CAR+ NK-92 cells [%]
MOCK
NFATsyn.EGFP. G$_{D2}$CAR
NFATmIL2.EGFP. G$_{D2}$CAR
NFκBsyn.EGFP. G$_{D2}$CAR
NFκBmIL2.EGFP. G$_{D2}$CAR
KG-1α
KG-1α GD2
NFκB-driven EGFP expression in G$_{D2}$CAR+ primary NK cells [%]
NFκBsyn. NFκBmIL2. EGFP G$_{D2}$CAR
patient-derived glioblastoma
G$_{D2}$CAR
EGFP
NFκBsyn. EGFP.G$_{D2}$CAR
NFκBmIL2. EGFP.G$_{D2}$CAR
NFκBsyn. EGFP.G$_{D2}$CAR
NFκBmIL2.

REFERENCES CITED IN THE DESCRIPTION

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