

ANTIGEN RECEPTORS AND USES THEREOF**TECHNICAL FIELD OF THE INVENTION**

The present invention relates to recombinant antigen receptors and uses thereof. T cells engineered to express such antigen receptors are useful in the treatment of diseases characterized by expression of one or more antigens bound by the antigen receptors.

BACKGROUND OF THE INVENTION

T cells play a central role in cell-mediated immunity in humans and animals. The recognition and binding of a particular antigen is mediated by the T cell receptors (TCRs) expressed on the surface of T cells. The TCR of a T cell is able to interact with immunogenic peptides (epitopes) bound to major histocompatibility complex (MHC) molecules and presented on the surface of target cells. Specific binding of the TCR triggers a signal cascade inside the T cell leading to proliferation and differentiation into a matured effector T cell.

The TCR is a part of a complex signaling machinery, which includes the heterodimeric complex of the TCR α - and β -chains, the co-receptor CD4 or CD8 and the CD3 signal transduction module (Figure 1). The TCR α/β heterodimer is responsible for antigen recognition and relaying the activation signal through the cell membrane in concert with CD3, while the CD3 chains themselves transfer the incoming signal to adaptor proteins inside the cell. Thus, the transfer of the TCR α/β chains offers the opportunity to redirect T cells towards any antigen of interest.

Adoptive cell transfer (ACT) based immunotherapy can be broadly defined as a form of passive immunization with previously sensitized T cells that are transferred to non-immune recipients or to the autologous host after *ex vivo* expansion from low precursor frequencies to clinically relevant cell numbers. Cell types that have been used for ACT experiments include lymphokine-activated killer (LAK) cells (Mule, J.J. et al. (1984) Science 225, 1487-1489; Rosenberg, S.A. et al. (1985) N. Engl. J. Med. 313, 1485-1492), tumor-infiltrating lymphocytes (TILs) (Rosenberg, S.A. et al. (1994) J. Natl. Cancer Inst. 86, 1159-1166), donor lymphocytes after hematopoietic stem cell transplantation (HSCT) as well as tumor-specific T cell lines or clones (Dudley, M.E. et al. (2001) J. Immunother. 24, 363-373; Yee, C. et al. (2002) Proc. Natl. Acad. Sci. U. S. A 99, 16168-16173). Adoptive T cell transfer was shown to have therapeutic activity against human

viral infections such as CMV. For adoptive immunotherapy of melanoma Rosenberg and co-workers established an ACT approach relying on the infusion of *in vitro* expanded autologous tumor-infiltrating lymphocytes (TILs) isolated from excised tumors in combination with a non-myeloablative lymphodepleting chemotherapy and high-dose IL2. A clinical study resulted in an objective response rate of ~50% of treated patients suffering from metastatic melanoma (Dudley, M.E. et al. (2005) J. Clin. Oncol. 23: 2346-2357).

An alternative approach is the adoptive transfer of autologous T cells reprogrammed to express a tumor-reactive immunoreceptor of defined specificity during short-time *ex vivo* culture followed by reinfusion into the patient (Kershaw M.H. et al. (2013) Nature Reviews Cancer 13 (8):525-41). This strategy makes ACT applicable to a variety of common malignancies even if tumor-reactive T cells are absent in the patient. Since the antigenic specificity of T cells is rested entirely on the heterodimeric complex of the TCR α - and β -chain, the transfer of cloned TCR genes into T cells offers the potential to redirect them towards any antigen of interest. Therefore, TCR gene therapy provides an attractive strategy to develop antigen-specific immunotherapy with autologous lymphocytes as treatment option. Major advantages of TCR gene transfer are the creation of therapeutic quantities of antigen-specific T cells within a few days and the possibility to introduce specificities that are not present in the endogenous TCR repertoire of the patient. Several groups demonstrated, that TCR gene transfer is an attractive strategy to redirect antigen-specificity of primary T cells (Morgan, R.A. et al. (2003) J. Immunol. 171, 3287-3295; Cooper, L.J. et al. (2000) J. Virol. 74, 8207-8212; Fujio, K. et al. (2000) J. Immunol. 165, 528-532; Kessels, H.W. et al. (2001) Nat. Immunol. 2, 957-961; Dembic, Z. et al. (1986) Nature 320, 232-238). Feasibility of TCR gene therapy in humans was initially demonstrated in clinical trials for the treatment of malignant melanoma by Rosenberg and his group. The adoptive transfer of autologous lymphocytes retrovirally transduced with melanoma/melanocyte antigen-specific TCRs resulted in cancer regression in up to 30% of treated melanoma patients (Morgan, R.A. et al. (2006) Science 314, 126-129; Johnson, L.A. et al. (2009) Blood 114, 535-546). In the meantime clinical testing of TCR gene therapy was extended also to cancers other than melanoma targeting many different tumor antigens (Park, T.S. et al., (2011) Trends Biotechnol. 29, 550-557).

The use of genetic engineering approaches to insert antigen-targeted receptors of defined specificity into T cells has greatly extended the potential capabilities of ACT. Chimeric antigen receptors (CARs) are a type of antigen-targeted receptor composed of intracellular T cell

signaling domains fused to extracellular antigen-binding domains, most commonly single-chain variable fragments (scFv's) from monoclonal antibodies. CARs directly recognize cell surface antigens, independent of MHC-mediated presentation, permitting the use of a single receptor construct specific for any given antigen in all patients. Initial CARs fused antigen-recognition domains to the CD3 ζ activation chain of the T cell receptor (TCR) complex (Figure 2). Subsequent CAR iterations have included secondary costimulatory signals in tandem with CD3 ζ , including intracellular domains from CD28 or a variety of TNF receptor family molecules such as 4-1BB (CD137) and OX40 (CD134). Further, third generation receptors include two costimulatory signals in addition to CD3 ζ , most commonly from CD28 and 4-1BB. Second and third generation CARs dramatically improved antitumor efficacy *in vitro* and *in vivo* (Zhao et al., (2009) J. Immunol., (183) 5563-5574), in some cases inducing complete remissions in patients with advanced cancer (Porter et al., (2011) N.Engl.J.Med., (365) 725-733) .

A classical CAR consists of an antigen-specific single chain antibody (scFv) fragment, fused to a transmembrane and signaling domain such as CD3 ζ . Upon introduction into T cells it is expressed as a membrane-bound protein and induces immune responses upon binding to its cognate antigen (Eshhar et al., (1993) PNAS, (90) 720-724). The induced antigen-specific immune response results in the activation of cytotoxic CD8⁺ T cells which in turn leads to the eradication of cells expressing the specific antigen, such as tumor cells or virus-infected cells expressing the specific antigen. However, these classical CAR constructs do not activate/stimulate the T cells through their endogenous CD3 complex, which is normally essential for T cell activation. Due to the fusion of the antigen binding domain to CD3 ζ , T cell activation is induced through a biochemical "short circuit" (Aggen et al., (2012) Gene Therapy, (19) 365-374). This non-physiological activation of T cells through this biochemical short circuit is risky for the patient being treated this way since over-activation of T cells may lead to unwanted side effects. For example, long-term basal activation of recombinant T cells due to CAR expression has been observed *in vitro* ("tonic signaling"), which resulted in an increased accumulation of inhibitory molecules, such as LAG-3, TIM-3 and PD-1, on the surface of recombinant CAR-expressing T cells, which in turn resulted in prematurely exhausted T-cells subsequently leading to a strong negative impact on the response against tumor cells *in vivo* (Long et al., (2015) Nat. Med., (21) 581-590). This adverse reaction has been associated with an irregular clustering of scFv-fragments through framework residues of this antibody. Additionally, while classic CAR constructs of this type have been successfully tested against different neoplasias, such as leukemia (Porter et al., (2011) N.Engl.J.Med., (365) 725-733), they

have also resulted in fatal autoimmune diseases due to basal expression of the targeted antigen (targeted tumor antigen) in normal tissues (on-target/off-tumor-reaction; Morgan et al., (2010) *Mol Ther.*, (18) 843-51).

An alternative approach, in which activation of the T cell occurs through a more physiological mechanism, was the provision of an analogous single chain-TCR (scTv)-fragment fused to the C β constant domain derived from the T cell receptor (TCR) and its co-expression with a TCR-derived C α constant domain (Voss et al., (2010) *Blood*, (115) 5154-5163), the latter which recruits the essential endogenous CD3 ζ homodimer (Call et al., (2002) *Cell*, (111) 967-79.). However, in order for these constructs to function as immune system activators, it was essential that the constant domains derived from the TCR be derived from mice TCRs or be murinized (Cohen et al., (2006) *Cancer Res.*, (66) 8878-86) to achieve chain pairing between the scTCR and C α . The fact that these constructs must have xenogenic sequences for functionality heightens the risk that the immune system will react against them when administered and lessens or destroys their therapeutic effectiveness.

Thus, there is a need for the provision of alternate recombinant antigen receptors, in which, e.g., the receptor, upon antigen binding, is sufficiently able to activate the T cell in which it is expressed in a normal physiological manner through the endogenous CD3 complex and, optionally, with no requirement for the presence of any amino acid sequences not of human origin, at least in the signal transmission domain of the antigen receptor, that could induce an unwanted immune response against the recombinant antigen receptor itself.

DESCRIPTION OF INVENTION

Summary

The present disclosure relates to recombinant antigen receptors having at least two antigen binding sites. The antigen receptors comprise two peptide chains. In one aspect, the present disclosure refers to an antigen receptor, which receptor comprises a first peptide chain and a second peptide chain, wherein the first peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; the second peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; wherein the immunoreceptor signal transmission domain is preferably of human origin, wherein the first

domain from the first peptide chain forms together with one of the domains from the second peptide chain a first antigen binding site, and wherein the second domain from the first peptide chain forms together with the other domain from the second peptide chain a second antigen binding site.

In another aspect, the present disclosure refers to a peptide chain comprising a first and a second domain which each comprise a variable region of a heavy chain of an immunoglobulin or each comprise a variable region of a light chain of an immunoglobulin and wherein the peptide chain further comprises an immunoreceptor signal transmission domain. In yet another aspect, the present disclosure refers to a recombinant cell expressing both the first and second peptide chains as described herein. In yet another aspect, the present disclosure refers to a recombinant cell expressing the peptide chain as described herein. In yet another aspect, the present disclosure refers to an *ex vivo* method for producing a cell expressing an antigen receptor which receptor comprises a first peptide chain and a second peptide chain, the method comprising: (a) providing a cell, preferably a human cell; (b) providing a first genetic construct encoding the first peptide chain comprising at least a first and a second domain, and an immunoreceptor signal transmission domain; (c) providing a second genetic construct encoding the second peptide chain comprising at least a first and a second domain and an immunoreceptor signal transmission domain; (d) introducing the first and second genetic constructs into the cell; and (e) allowing the constructs to be expressed in the cell; wherein the cell is preferably a T cell, wherein the first domain from the first peptide chain is able to form together with one of the domains from the second peptide chain a first antigen binding site, and wherein the second domain from the first peptide chain is able to form together with the other domain from the second peptide chain a second antigen binding site, wherein the first peptide chain and the second peptide chain are preferably provided on a single genetic construct.

In a further aspect, the present disclosure refers to a nucleic acid encoding both the first and second peptide chains as described herein, wherein the nucleic acid is preferably DNA or RNA. In yet another aspect, the present disclosure refers to a nucleic acid encoding the peptide chain as described herein, wherein the nucleic acid is preferably DNA or RNA. In yet another aspect, the present disclosure refers to a pharmaceutical composition comprising (a) the antigen receptor as described herein, (b) the recombinant cell as described herein, (c) a nucleic acid as described herein, or (d) a nucleic acid encoding the first peptide chain *and* a nucleic acid encoding the

second peptide chain, said chains being defined herein; and a pharmaceutically acceptable carrier, for use as a medicament.

In yet another aspect, the present disclosure refers to the use of the antigen receptor as described herein, the recombinant cell as described herein, or the nucleic acid as described herein; and a pharmaceutically acceptable carrier in the manufacture of a pharmaceutical composition for treating cancer.

In one example, the peptide chains each comprise at least two domains, in addition to an immunoreceptor signal transmission domain, in which each of the two domains on one peptide chain forms an antigen binding site with one of the domains on the other peptide chain. In another example, one of the peptide chains has at least four domains, in addition to an immunoreceptor signal transmission domain, in which two of the four domains form two antigen binding sites with the two remaining domains on the same peptide chain.

In one example, the present disclosure relates to an antigen receptor, which receptor comprises a first peptide chain and a second peptide chain, wherein the first peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; the second peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; wherein the first domain from the first peptide chain forms together with one of the domains from the second peptide chain a first antigen binding site, and wherein the second domain from the first peptide chain forms together with the other domain from the second peptide chain a second antigen binding site. In the antigen receptor of this example, the domains forming the respective antigen binding sites are preferably located on different peptide chains. Consequently, antigen binding sites are formed by intermolecular interaction of domains.

In one example, the first and/or second domains each comprise a variable region of an immunoglobulin chain or a variable region of a T cell receptor chain or a portion of the variable region.

In one example, one of the domains forming the first antigen binding site comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the other domain forming the first antigen binding site comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof. In one

example, one of the domains forming the second antigen binding site comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the other domain forming the second antigen binding site comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof.

In one example, the first domain from the first peptide chain comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the domain from the second peptide chain forming an antigen binding site with the first domain from the first peptide chain comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof. In one example, the second domain from the first peptide chain comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the domain from the second peptide chain forming an antigen binding site with the second domain from the first peptide chain comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof.

In one example, the first and the second domains from the first peptide chain each comprise a variable region of a heavy chain of an immunoglobulin or a portion thereof; and the first and the second domains from the second peptide chain each comprise a variable region of a light chain of an immunoglobulin or a portion thereof.

In one example, the N-terminal domain from the first peptide chain forms together with the N-terminal domain from the second peptide chain an antigen binding site; and the C-terminal domain from the first peptide chain forms together with the C-terminal domain from the second peptide chain an antigen binding site.

In one example, the N-terminal domain from the first peptide chain forms together with the C-terminal domain from the second peptide chain an antigen binding site; and the C-terminal domain from the first peptide chain forms together with the N-terminal domain from the second peptide chain an antigen binding site.

In one example, the present disclosure relates to an antigen receptor, which receptor comprises a first peptide chain and a second peptide chain, wherein the first peptide chain comprises at least four domains, and an immunoreceptor signal transmission domain; the second peptide chain

comprises an immunoreceptor signal transmission domain; wherein two of the domains from the first peptide chain form a first antigen binding site, and wherein the other two domains from the first peptide chain form a second antigen binding site. In the antigen receptor of this example, the domains forming the respective antigen binding sites are preferably located on the same peptide chain. Consequently, antigen binding sites are formed by intramolecular interaction of domains.

In one example, the four domains each comprise a variable region of an immunoglobulin chain or a variable region of a T cell receptor chain or a portion of the variable region.

In one example, one of the domains forming the first antigen binding site comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the other domain forming the first antigen binding site comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof. In one example, one of the domains forming the second antigen binding site comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the other domain forming the second antigen binding site comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof.

In one example, the two N-terminal domains of the four domains form together an antigen binding site; and the two C-terminal domains of the four domains form together an antigen binding site.

In one example, one of the two N-terminal domains of the four domains comprises a variable region of a heavy chain of an immunoglobulin or a portion thereof and the other of the two N-terminal domains of the four domains comprises a variable region of a light chain of an immunoglobulin or a portion thereof; and one of the two C-terminal domains of the four domains comprises a variable region of a heavy chain of an immunoglobulin or a portion thereof and the other of the two C-terminal domains of the four domains comprises a variable region of a light chain of an immunoglobulin or a portion thereof.

In one example of the antigen receptors of the disclosure, the immunoreceptor signal transmission domain comprises a constant or invariant region of a T cell receptor chain or a constant or invariant region of an immune cell Fc receptor chain or a portion of the constant or invariant region. In one example of the antigen receptors of the disclosure, (i) the first peptide

chain comprises a constant region of a T cell receptor alpha chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof, or (ii) the first peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof.

In one example of the antigen receptors of the disclosure, the immunoreceptor signal transmission domain is of human origin.

In one example, an antigen receptor of the disclosure comprises (a) linker(s) connecting domains of the antigen receptor. In one example, an antigen receptor of the disclosure comprises one or more linkers between the domains forming the antigen binding sites and/or between the domains forming the antigen binding sites and the immunoreceptor signal transmission domains. The linker can be an arbitrary amino acid sequence of any length so long as it does not interfere with the functions of the antigen receptor, such as the ability of the antigen receptor to bind antigen or to associate with the endogenous CD3 complex, or interfere with the ability of the antigen receptor to induce an immune response upon antigen binding.

In one example of the antigen receptors of the disclosure, the first and second antigen binding sites bind to the same antigen or different antigens. In one example of the antigen receptors of the disclosure, the first and second antigen binding sites bind to different epitopes on the same antigen. Consequently, while the domains forming the first antigen binding site are preferably derived from the same immunoglobulin and the domains forming the second antigen binding site are preferably derived from the same immunoglobulin, the domains forming the first antigen binding site and the domains forming the second antigen binding site are derived from the same or different immunoglobulins, said different immunoglobulins binding to the same or different antigens.

In one example, the antigen is a disease-specific antigen, preferably a tumor antigen. In one example, the antigen is expressed on the surface of a cell.

In one example, the present disclosure relates to a peptide chain of any of the antigen receptors of the disclosure. In one example, the present disclosure relates to a peptide chain comprising a first and a second domain which each comprise a variable region of a heavy chain of an

immunoglobulin or a portion thereof or each comprise a variable region of a light chain of an immunoglobulin or a portion thereof and wherein the peptide chain further comprises an immunoreceptor signal transmission domain. In one example, the present disclosure relates to a peptide chain which comprises at least four domains, and an immunoreceptor signal transmission domain, wherein two of the domains from the peptide chain form a first antigen binding site, and the other two domains from the peptide chain form a second antigen binding site. Further examples of the peptide chains of the disclosure are as described herein for the antigen receptors of the disclosure.

In one example, the present disclosure relates to a cell, in particular an immune effector cell such as a T cell, genetically modified to express an antigen receptor of the disclosure. In one example, the present disclosure relates to a recombinant cell, in particular an immune effector cell such as a T cell, expressing the first peptide chain, the second peptide chain or both the first and second peptide chains of an antigen receptor of the disclosure or expressing a peptide chain of the disclosure. Further examples of the cell or recombinant cell of the disclosure are as described herein for the antigen receptors of the disclosure or the peptide chains of the disclosure.

In one example, the present disclosure relates to a method for producing a cell expressing an antigen receptor of the disclosure, the method comprising: (a) providing a cell; (b) providing a first genetic construct encoding the first peptide chain of an antigen receptor of the disclosure; (c) providing a second genetic construct encoding the second peptide chain of an antigen receptor of the disclosure; (d) introducing the first and second genetic constructs into the cell; and (e) allowing the constructs to be expressed in the cell. In one example, the present disclosure relates to a method for producing a cell expressing an antigen receptor which receptor comprises a first peptide chain and a second peptide chain, the method comprising: (a) providing a cell; (b) providing a first genetic construct encoding the first peptide chain comprising at least a first and a second domain, and an immunoreceptor signal transmission domain; (c) providing a second genetic construct encoding the second peptide chain comprising at least a first and a second domain and an immunoreceptor signal transmission domain; (d) introducing the first and second genetic constructs into the cell; and (e) allowing the constructs to be expressed in the cell; wherein the first domain from the first peptide chain is able to form together with one of the domains from the second peptide chain a first antigen binding site, and wherein the second domain from the first peptide chain is able to form together with the other domain from the second peptide chain a second antigen binding site. In one example, the present disclosure relates

to a method for producing a cell expressing an antigen receptor which receptor comprises a first peptide chain and a second peptide chain, the method comprising: (a) providing a cell; (b) providing a first genetic construct encoding the first peptide chain comprising at least four domains, and an immunoreceptor signal transmission domain; (c) providing a second genetic construct encoding the second peptide chain comprising an immunoreceptor signal transmission domain; (d) introducing the first and second genetic constructs into the cell; and (e) allowing the constructs to be expressed in the cell; wherein two of the domains from the first peptide chain are able to form a first antigen binding site, and wherein the other two domains from the first peptide chain are able to form a second antigen binding site. In one example of the methods of the disclosure, expression of the antigen receptor is at the cell surface. In one example of the methods of the disclosure, the first peptide chain and the second peptide chain are provided on a single genetic construct. In one example of the methods of the disclosure, the cell is a human cell. In one example of the methods of the disclosure, the cell is an immune effector cell such as a T cell. In one example of the methods of the disclosure, the genetic constructs comprise DNA and/or RNA. Further examples of the methods of the disclosure are as described herein for the antigen receptors of the disclosure.

In one example, the present disclosure relates to a recombinant cell, in particular an immune effector cell such as a T cell, produced by the methods of the disclosure for producing a cell expressing an antigen receptor. Further examples of the recombinant cell of the disclosure are as described herein for the antigen receptors of the disclosure or the methods of the disclosure for producing a cell expressing an antigen receptor.

In one example, the present disclosure relates to a nucleic acid such as DNA or RNA encoding the first peptide chain, the second peptide chain or both the first and second peptide chains of an antigen receptor of the disclosure or encoding a peptide chain of the disclosure. Further examples of the nucleic acid of the disclosure are as described herein for the antigen receptors of the disclosure or the peptide chains of the disclosure.

The present disclosure generally embraces the treatment of diseases by targeting cells expressing one or more antigens on the cell surface such as diseased cells expressing one or more disease-specific antigens on the cell surface, in particular cancer cells expressing one or more tumor antigens on the cell surface using antigen receptors of the disclosure. The methods provide for the selective eradication of cells that express on their surface one or more antigens, thereby

minimizing adverse effects to normal cells not expressing the antigen(s). In one example, T cells genetically modified to express an antigen receptor of the disclosure targeting the cells through binding to the antigen(s) are administered. T cells are able to recognize diseased cells expressing the antigen(s) on the cell surface, resulting in the eradication of diseased cells. In one example, the target cell population or target tissue is tumor cells or tumor tissue.

In one example, the present disclosure relates to a pharmaceutical composition comprising the antigen receptor of the disclosure, the recombinant cell of the disclosure, or the nucleic acid of the disclosure; and a pharmaceutically acceptable carrier. The pharmaceutical composition of the disclosure may be used as a medicament, in particular in the treatment of a disease such as cancer characterized by expression of one or more antigens which are bound by the antigen receptor of the disclosure such as one or more tumor antigens.

In one example, the present disclosure relates to a method of treating a disease such as cancer comprising administering to a subject a therapeutically effective amount of the pharmaceutical composition of the disclosure, wherein the disease is characterized by expression of at least one antigen such as a tumor antigen which is bound by the antigen receptor.

In one example, the present disclosure relates to a method of treating a subject having a disease, disorder or condition associated with expression or elevated expression of at least one antigen, the method comprising administering to the subject T cells genetically modified to express an antigen receptor of the disclosure targeted to the at least one antigen. In one example, the disease, disorder or condition is cancer. In one example, the T cells may be autologous, allogeneic or syngeneic to the subject.

In one example of the disclosure, the antigen receptor binds to only one antigen (e.g. by being monospecific and recognizing the same epitope or by being bispecific or multispecific and recognizing different epitopes on the same antigen) or binds to different antigens, in particular two different antigens.

Further described in the disclosure is the method of treating further comprises obtaining a sample of cells from a subject, the sample comprising T cells or T cell progenitors, and transfecting the cells with a nucleic acid encoding the antigen receptor of the disclosure to provide T cells genetically modified to express the antigen receptor. Further described in the disclosure is the T

cells genetically modified to express the antigen receptor are stably or transiently transfected with nucleic acid encoding the antigen receptor. Thus, the nucleic acid encoding the antigen receptor is integrated or not integrated into the genome of the T cells. Further described in the disclosure is the T cells and/or the sample of cells are from the subject to which the T cells genetically modified to express the antigen receptor are administered. Further described in the disclosure is the T cells and/or the sample of cells are from a mammal which is different to the mammal to which the T cells genetically modified to express the antigen receptor are administered.

Also described in the disclosure is the T cells genetically modified to express the antigen receptor are inactivated for expression of an endogenous T cell receptor and/or endogenous HLA.

Also described in the disclosure is an antigen is expressed in a diseased cell such as a cancer cell. In one example, an antigen is expressed on the surface of a diseased cell such as a cancer cell. In one example, an antigen receptor binds to an extracellular domain or to an epitope in an extracellular domain of an antigen. In one example, an antigen receptor binds to native epitopes of an antigen present on the surface of living cells. Also described in the disclosure is the antigen is a tumor antigen. Also described in the disclosure is the antigen is selected from the group consisting of claudins, such as claudin 6 and claudin 18.2, CD19, CD20, CD22, CD33, CD123, mesothelin, CEA, c-Met, PSMA, GD-2, and NY-ESO-1. Also described in the disclosure is the antigen is a pathogen antigen. The pathogen may be a fungal, viral, or bacterial pathogen. Further described in the disclosure is expression of the antigen is at the cell surface. In one example an antigen is a claudin, in particular claudin 6 or claudin 18.2, and said antigen receptor binds to the first extracellular loop of said claudin. In one example, binding of said antigen receptor when expressed by T cells and/or present on T cells to an antigen present on cells results in immune effector functions of said T cells such as the release of cytokines. In one example, binding of said antigen receptor when expressed by T cells and/or present on T cells to an antigen present on cells such as antigen presenting cells results in stimulation, priming and/or expansion of said T cells. In one example, binding of said antigen receptor when expressed by T cells and/or present on T cells to an antigen present on diseased cells such as cancer cells results in cytolysis and/or apoptosis of the diseased cells, wherein said T cells preferably release cytotoxic factors, e.g. perforins and granzymes.

Also described in the disclosure is the domains of an antigen receptor forming antigen binding sites are comprised by an exodomain of the antigen receptor. Also described in the disclosure is an antigen receptor of the disclosure comprises a transmembrane domain. In one example, the transmembrane domain is a hydrophobic alpha helix that spans the membrane.

Also described in the disclosure is an antigen receptor of the disclosure comprises a signal peptide which directs the nascent protein into the endoplasmic reticulum. In one example, the signal peptide precedes the domains forming antigen binding sites.

Also described in the disclosure is an antigen receptor of the disclosure is preferably specific for the antigen to which it is targeted, in particular when present on the surface of a cell such as a diseased cell or an antigen-presenting cell.

Also described in the disclosure is an antigen receptor of the disclosure may be expressed by and/or present on the surface of a T cell, preferably a cytotoxic T cell. In one example, the T cell is reactive with the antigen(s) to which the an antigen receptor of the disclosure is targeted.

In a further example, the disclosure provides the agents and compositions described herein for use in the methods described herein.

Other features and advantages of the instant disclosure will be apparent from the following detailed description and claims.

Detailed description of the invention

Although the present invention is described in detail below, it is to be understood that this invention is not limited to the particular methodologies, protocols and reagents described herein as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims. Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art.

In the following, the elements of the present invention will be described. These elements are listed with specific embodiments, however, it should be understood that they may be combined in any manner and in any number to create additional embodiments. The variously described examples and preferred embodiments should not be construed to limit the present invention to only the explicitly described embodiments. This description should be understood to support and

encompass embodiments which combine the explicitly described embodiments with any number of the disclosed and/or preferred elements. Furthermore, any permutations and combinations of all described elements in this application should be considered disclosed by the description of the present application unless the context indicates otherwise.

Preferably, the terms used herein are defined as described in "A multilingual glossary of biotechnological terms: (IUPAC Recommendations)", H.G.W. Leuenberger, B. Nagel, and H. Kölbl, Eds., (1995) Helvetica Chimica Acta, CH-4010 Basel, Switzerland.

The practice of the present invention will employ, unless otherwise indicated, conventional methods of biochemistry, cell biology, immunology, and recombinant DNA techniques which are explained in the literature in the field (cf., e.g., *Molecular Cloning: A Laboratory Manual*, 2nd Edition, J. Sambrook et al. eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor 1989).

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated member, integer or step or group of members, integers or steps but not the exclusion of any other member, integer or step or group of members, integers or steps although in some embodiments such other member, integer or step or group of members, integers or steps may be excluded, i.e. the subject-matter consists in the inclusion of a stated member, integer or step or group of members, integers or steps. The terms "a" and "an" and "the" and similar reference used in the context of describing the invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context. Recitation of ranges of values herein is merely intended to serve as a shorthand method of referring individually to each separate value falling within the range. Unless otherwise indicated herein, each individual value is incorporated into the specification as if it were individually recited herein.

All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise 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 illustrate the invention and does not pose a limitation on the scope of the invention otherwise claimed. No

language in the specification should be construed as indicating any non-claimed element essential to the practice of the invention.

Several documents are cited throughout the text of this specification. Each of the documents cited herein (including all patents, patent applications, scientific publications, manufacturer's specifications, instructions, etc.), whether *supra* or *infra*, are hereby incorporated by reference in their entirety. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of prior invention.

The term "immune response" refers to an integrated bodily response to an antigen and preferably refers to a cellular immune response or a cellular as well as a humoral immune response. The immune response may be protective/preventive/prophylactic and/or therapeutic.

"Providing an immune response" may mean that there was no immune response against a particular target antigen, target cell and/or target tissue before providing an immune response, but it may also mean that there was a certain level of immune response against a particular target antigen, target cell and/or target tissue before providing an immune response and after providing an immune response said immune response is enhanced. Thus, "providing an immune response" includes "inducing an immune response" and "enhancing an immune response". Preferably, after providing an immune response in a subject, said subject is protected from developing a disease such as a cancer disease or the disease condition is ameliorated by providing an immune response. For example, an immune response against a tumor antigen may be provided in a patient having a cancer disease or in a subject being at risk of developing a cancer disease. Providing an immune response in this case may mean that the disease condition of the subject is ameliorated, that the subject does not develop metastases, or that the subject being at risk of developing a cancer disease does not develop a cancer disease.

"Cell-mediated immunity" or "cellular immunity", or similar terms are meant to include a cellular response directed to cells characterized by expression of an antigen, in particular characterized by presentation of an antigen with class I or class II MHC. The cellular response relates to cells called T cells or T-lymphocytes which act as either "helpers" or "killers". The helper T cells (also termed CD4⁺ T cells) play a central role by regulating the immune response and the killer cells (also termed cytotoxic T cells, cytolytic T cells, CD8⁺ T cells or CTLs) kill diseased cells such as cancer cells, preventing the production of more diseased cells.

The term "antigen" relates to an agent comprising an epitope against which an immune response is to be generated and/or is directed. Preferably, an antigen in the context of the present invention is a molecule which, optionally after processing, induces an immune reaction, which is preferably specific for the antigen or cells expressing the antigen, preferably on the cell surface. The term "antigen" includes in particular proteins and peptides. An antigen is preferably a product which corresponds to or is derived from a naturally occurring antigen. Such naturally occurring antigens may include or may be derived from allergens, viruses, bacteria, fungi, parasites and other infectious agents and pathogens or an antigen may also be a tumor antigen. According to the present invention, an antigen may correspond to a naturally occurring product, for example, a viral protein, or a part thereof.

The term "pathogen" relates to pathogenic microorganisms and comprises viruses, bacteria, fungi, unicellular organisms, and parasites. Examples for pathogenic viruses are human immunodeficiency virus (HIV), cytomegalovirus (CMV), herpes virus (HSV), hepatitis A-virus (HAV), HBV, HCV, papilloma virus, and human T-lymphotrophic virus (HTLV). Unicellular organisms comprise plasmodia, trypanosomes, amoeba, etc.

In a preferred embodiment, an antigen is a disease-specific antigen or disease-associated antigen. The term "disease-specific antigen" or "disease-associated antigen" refers to all antigens that are of pathological significance. In one particularly preferred embodiment, the antigen is present in diseased cells, tissues and/or organs while it is not present or present in a reduced amount in healthy cells, tissues and/or organs and, thus, can be used for targeting diseased cells, tissues and/or organs, e.g. by T cells carrying an antigen receptor targeted to the antigen. In one embodiment, a disease-specific antigen or disease-associated antigen is present on the surface of a diseased cell.

In a preferred embodiment, an antigen is a tumor antigen or tumor-associated antigen, i.e., a constituent of cancer cells which may be derived from the cytoplasm, the cell surface and the cell nucleus, in particular those antigens which are produced, preferably in large quantity, as surface antigens on cancer cells.

In the context of the present invention, the term "tumor antigen" or "tumor-associated antigen" relates to proteins that are under normal conditions specifically expressed in a limited number of

tissues and/or organs or in specific developmental stages, for example, the tumor antigen may be under normal conditions specifically expressed in stomach tissue, preferably in the gastric mucosa, in reproductive organs, e.g., in testis, in trophoblastic tissue, e.g., in placenta, or in germ line cells, and are expressed or aberrantly expressed in one or more tumor or cancer tissues. In this context, "a limited number" preferably means not more than 3, more preferably not more than 2. The tumor antigens in the context of the present invention include, for example, differentiation antigens, preferably cell type specific differentiation antigens, i.e., proteins that are under normal conditions specifically expressed in a certain cell type at a certain differentiation stage, cancer/testis antigens, i.e., proteins that are under normal conditions specifically expressed in testis and sometimes in placenta, and germ line specific antigens. In the context of the present invention, the tumor antigen is preferably associated with the cell surface of a cancer cell and is preferably not or only rarely expressed in normal tissues. Preferably, the tumor antigen or the aberrant expression of the tumor antigen identifies cancer cells. In the context of the present invention, the tumor antigen that is expressed by a cancer cell in a subject, e.g., a patient suffering from a cancer disease, is preferably a self-protein in said subject. In preferred embodiments, the tumor antigen in the context of the present invention is expressed under normal conditions specifically in a tissue or organ that is non-essential, i.e., tissues or organs which when damaged by the immune system do not lead to death of the subject, or in organs or structures of the body which are not or only hardly accessible by the immune system. Preferably, the amino acid sequence of the tumor antigen is identical between the tumor antigen which is expressed in normal tissues and the tumor antigen which is expressed in cancer tissues.

Examples for tumor antigens that may be useful in the present invention are p53, ART-4, BAGE, beta-catenin/m, Bcr-abL CAMEL, CAP-1, CASP-8, CDC27/m, CDK4/m, CEA, the cell surface proteins of the claudin family, such as CLAUDIN-6, CLAUDIN-18.2 and CLAUDIN-12, c-MYC, CT, Cyp-B, DAM, ELF2M, ETV6-AML1, G250, GAGE, GnT-V, Gap100, HAGE, HER-2/neu, HPV-E7, HPV-E6, HAST-2, hTERT (or hTRT), LAGE, LDLR/FUT, MAGE-A, preferably MAGE-A1, MAGE-A2, MAGE-A3, MAGE-A4, MAGE-A5, MAGE-A6, MAGE-A7, MAGE-A8, MAGE-A9, MAGE-A10, MAGE-A11, or MAGE-A12, MAGE-B, MAGE-C, MART-1/Melan-A, MC1R, Myosin/m, MUC1, MUM-1, -2, -3, NA88-A, NF1, NY-ESO-1, NY-BR-1, p190 minor BCR-abL, Pm1/RARa, PRAME, proteinase 3, PSA, PSM, RAGE, RU1 or RU2, SAGE, SART-1 or SART-3, SCGB3A2, SCP1, SCP2, SCP3, SSX, SURVIVIN, TEL/AML1, TPI/m, TRP-1, TRP-2, TRP-2/INT2, TPTE and WT. Particularly preferred tumor antigens include CLAUDIN-18.2 (CLDN18.2) and CLAUDIN-6 (CLDN6).

The term "CLDN" or simply "Cl" as used herein means claudin and includes CLDN6 and CLDN18.2. Preferably, a claudin is a human claudin. Claudins are a family of proteins that are the most important components of tight junctions, where they establish the paracellular barrier that controls the flow of molecules in the intercellular space between cells of an epithelium. Claudins are transmembrane proteins spanning the membrane 4 times with the N-terminal and the C-terminal end both located in the cytoplasm. The first extracellular loop, termed EC1 or ECL1, consists on average of 53 amino acids, and the second extracellular loop, termed EC2 or ECL2, consists of around 24 amino acids. Cell surface proteins of the claudin family are expressed in tumors of various origins, and are particularly suited as target structures in connection with targeted cancer immunotherapy due to their selective expression (no expression in a toxicity relevant normal tissue) and localization to the plasma membrane.

CLDN6 and CLDN18.2 have been identified as differentially expressed in tumor tissues, with the only normal tissue expressing CLDN18.2 being stomach (differentiated epithelial cells of the gastric mucosa) and the only normal tissue expressing CLDN6 being placenta.

CLDN18.2 is expressed in cancers of various origins such as pancreatic carcinoma, esophageal carcinoma, gastric carcinoma, bronchial carcinoma, breast carcinoma, and ENT tumors. CLDN18.2 is a valuable target for the prevention and/or treatment of primary tumors, such as gastric cancer, esophageal cancer, pancreatic cancer, lung cancer such as non small cell lung cancer (NSCLC), ovarian cancer, colon cancer, hepatic cancer, head-neck cancer, and cancers of the gallbladder, and metastases thereof, in particular gastric cancer metastasis such as Krukenberg tumors, peritoneal metastasis, and lymph node metastasis. Antigen receptors targeting at least CLDN18.2 are useful in treating such cancer diseases.

CLDN6 has been found to be expressed, for example, in ovarian cancer, lung cancer, gastric cancer, breast cancer, hepatic cancer, pancreatic cancer, skin cancer, melanomas, head neck cancer, sarcomas, bile duct cancer, renal cell cancer, and urinary bladder cancer. CLDN6 is a particularly preferred target for the prevention and/or treatment of ovarian cancer, in particular ovarian adenocarcinoma and ovarian teratocarcinoma, lung cancer, including small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), in particular squamous cell lung carcinoma and adenocarcinoma, gastric cancer, breast cancer, hepatic cancer, pancreatic cancer, skin cancer, in particular basal cell carcinoma and squamous cell carcinoma, malignant

melanoma, head and neck cancer, in particular malignant pleomorphic adenoma, sarcoma, in particular synovial sarcoma and carcinosarcoma, bile duct cancer, cancer of the urinary bladder, in particular transitional cell carcinoma and papillary carcinoma, kidney cancer, in particular renal cell carcinoma including clear cell renal cell carcinoma and papillary renal cell carcinoma, colon cancer, small bowel cancer, including cancer of the ileum, in particular small bowel adenocarcinoma and adenocarcinoma of the ileum, testicular embryonal carcinoma, placental choriocarcinoma, cervical cancer, testicular cancer, in particular testicular seminoma, testicular teratoma and embryonic testicular cancer, uterine cancer, germ cell tumors such as a teratocarcinoma or an embryonal carcinoma, in particular germ cell tumors of the testis, and the metastatic forms thereof. Antigen receptors targeting at least CLDN6 are useful in treating such cancer diseases.

In the context of the embodiments of the present invention, an antigen is preferably present on the surface of a cell, preferably an antigen presenting cell or diseased cell. According to the invention, an antigen if bound by an antigen receptor is preferably able to induce, optionally in the presence of appropriate co-stimulatory signals, stimulation, priming and/or expansion of the T cell carrying the antigen receptor binding the antigen. Recognition of an antigen on the surface of a diseased cell may result in an immune reaction against the antigen (or cell expressing the antigen).

According to the various aspects of the invention, the aim is preferably to provide an immune response against diseased cells expressing an antigen such as cancer cells expressing an antigen such as a tumor antigen, in particular CLDN6 or CLDN18.2, and to treat a disease such as a cancer disease involving cells expressing an antigen such as a tumor antigen. Preferably the invention involves the administration of antigen receptor-engineered immune effector cells such as T cells targeted against diseased cells expressing an antigen. Cells expressing an antigen on the surface can be targeted by immune effector cells carrying an antigen receptor targeted to the antigen.

"Cell surface" is used in accordance with its normal meaning in the art, and thus includes the outside of the cell which is accessible to binding by proteins and other molecules. An antigen is expressed on the surface of cells if it is located at the surface of said cells and is accessible to binding by antigen-binding molecules such as antigen receptors or antigen-specific antibodies added to the cells. In one embodiment, an antigen expressed on the surface of cells is an integral

membrane protein having an extracellular portion recognized by an antigen receptor. An antigen receptor is expressed on the surface of cells if it is located at the surface of said cells and is accessible to binding by e.g. antigen to which the antigen receptor is specific added to the cells. In one embodiment, an antigen receptor expressed on the surface of cells is an integral membrane protein having an extracellular portion recognizing antigen.

The term "extracellular portion" or "exodomain" in the context of the present invention refers to a part of a molecule such as a protein that is facing the extracellular space of a cell and preferably is accessible from the outside of said cell, e.g., by binding molecules such as antibodies located outside the cell. Preferably, the term refers to one or more extracellular loops or domains or a fragment thereof.

The terms "portion" or "part" are used interchangeably herein and refer to a continuous or discontinuous element of a structure such as an amino acid sequence. The term "fragment" refers to a continuous element of a structure such as an amino acid sequence. A portion, part or fragment of a structure preferably comprises one or more functional properties, e.g. antigenic, immunologic and/or binding properties, of said structure. A portion or part of a protein sequence preferably comprises at least 6, in particular at least 8, at least 12, at least 15, at least 20, at least 30, at least 50, or at least 100 consecutive and/or non-consecutive amino acids of the protein sequence. A fragment of a protein sequence preferably comprises at least 6, in particular at least 8, at least 12, at least 15, at least 20, at least 30, at least 50, or at least 100 consecutive amino acids of the protein sequence

According to the invention, an antigen is not (substantially) expressed in a cell if the level of expression is below the detection limit and/or if the level of expression is too low to allow binding by antigen-specific antibodies added to the cell. According to the invention, an antigen is expressed in a cell if the level of expression is above the detection limit and/or if the level of expression is high enough to allow binding by antigen-specific antibodies added to the cell. Preferably, an antigen expressed in a cell is expressed or exposed, i.e. is present, on the surface of said cell and, thus, available for binding by antigen-specific molecules such as antibodies or antigen receptors added to the cell.

"Target cell" shall mean a cell which is a target for an immune response such as a cellular immune response. Target cells include any undesirable cell such as a cancer cell. In preferred

embodiments, the target cell is a cell expressing a target antigen, in particular a disease-specific antigen, which preferably is present on the cell surface.

The term "epitope" refers to an antigenic determinant in a molecule such as an antigen, i.e., to a part in or fragment of the molecule that is recognized, i.e. bound, by the immune system, for example, that is recognized by an antibody or antigen receptor. For example, epitopes are the discrete, three-dimensional sites on an antigen, which are recognized by the immune system. Epitopes usually consist of chemically active surface groupings of molecules such as amino acids or sugar side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics. Conformational and non-conformational epitopes are distinguished in that the binding to the former but not the latter is lost in the presence of denaturing solvents. Preferably an epitope is capable of eliciting an immune response against the antigen or a cell expressing the antigen. Preferably, the term relates to an immunogenic portion of an antigen. An epitope of a protein such as a tumor antigen preferably comprises a continuous or discontinuous portion of said protein and is preferably between 5 and 100, preferably between 5 and 50, more preferably between 8 and 30, most preferably between 10 and 25 amino acids in length, for example, the epitope may be preferably 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 amino acids in length.

"Antigen processing" refers to the degradation of an antigen into procession products, which are fragments of said antigen (e.g., the degradation of a protein into peptides) and the association of one or more of these fragments (e.g., via binding) with MHC molecules for presentation by cells, preferably antigen presenting cells to specific T cells.

An antigen-presenting cell (APC) is a cell that displays antigen in the context of major histocompatibility complex (MHC) on its surface. T cells may recognize this complex using their T cell receptor (TCR). Antigen-presenting cells process antigens and present them to T cells. According to the invention, the term "antigen-presenting cell" includes professional antigen-presenting cells and non-professional antigen-presenting cells.

Professional antigen-presenting cells are very efficient at internalizing antigen, either by phagocytosis or by receptor-mediated endocytosis, and then displaying a fragment of the antigen, bound to a class II MHC molecule, on their membrane. The T cell recognizes and interacts with the antigen-class II MHC molecule complex on the membrane of the antigen-presenting cell. An

additional co-stimulatory signal is then produced by the antigen-presenting cell, leading to activation of the T cell. The expression of co-stimulatory molecules is a defining feature of professional antigen-presenting cells. The main types of professional antigen-presenting cells are dendritic cells, which have the broadest range of antigen presentation, and are probably the most important antigen-presenting cells, macrophages, B-cells, and certain activated epithelial cells.

Non-professional antigen-presenting cells do not constitutively express the MHC class II proteins required for interaction with naive T cells; these are expressed only upon stimulation of the non-professional antigen-presenting cells by certain cytokines such as IFN γ .

Dendritic cells (DCs) are leukocyte populations that present antigens captured in peripheral tissues to T cells via both MHC class II and I antigen presentation pathways. It is well known that dendritic cells are potent inducers of immune responses and the activation of these cells is a critical step for the induction of antitumoral immunity. Dendritic cells and progenitors may be obtained from peripheral blood, bone marrow, tumor-infiltrating cells, peritumoral tissues-infiltrating cells, lymph nodes, spleen, skin, umbilical cord blood or any other suitable tissue or fluid. For example, dendritic cells may be differentiated *ex vivo* by adding a combination of cytokines such as GM-CSF, IL-4, IL-13 and/or TNF α to cultures of monocytes harvested from peripheral blood. Alternatively, CD34 positive cells harvested from peripheral blood, umbilical cord blood or bone marrow may be differentiated into dendritic cells by adding to the culture medium combinations of GM-CSF, IL-3, TNF α , CD40 ligand, LPS, flt3 ligand and/or other compound(s) that induce differentiation, maturation and proliferation of dendritic cells. Dendritic cells are conveniently categorized as "immature" and "mature" cells, which can be used as a simple way to discriminate between two well characterized phenotypes. However, this nomenclature should not be construed to exclude all possible intermediate stages of differentiation. Immature dendritic cells are characterized as antigen presenting cells with a high capacity for antigen uptake and processing, which correlates with the high expression of Fc γ receptor and mannose receptor. The mature phenotype is typically characterized by a lower expression of these markers, but a high expression of cell surface molecules responsible for T cell activation such as class I and class II MHC, adhesion molecules (e. g. CD54 and CD11) and costimulatory molecules (e. g., CD40, CD80, CD86 and 4-1 BB). Dendritic cell maturation is referred to as the status of dendritic cell activation at which such antigen-presenting dendritic cells lead to T cell priming, while presentation by immature dendritic cells results in tolerance. Dendritic cell maturation is chiefly caused by biomolecules with microbial features detected by

innate receptors (bacterial DNA, viral RNA, endotoxin, etc.), pro-inflammatory cytokines (TNF, IL-1, IFNs), ligation of CD40 on the dendritic cell surface by CD40L, and substances released from cells undergoing stressful cell death. The dendritic cells can be derived by culturing bone marrow cells *in vitro* with cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and tumor necrosis factor alpha.

The term "immunogenicity" relates to the relative efficiency of an antigen to induce an immune reaction.

The term "immune effector functions" in the context of the present invention includes any functions mediated by components of the immune system that result, for example, in the killing of diseased cells such as tumor cells, or in the inhibition of tumor growth and/or inhibition of tumor development, including inhibition of tumor dissemination and metastasis. Preferably, the immune effector functions in the context of the present invention are T cell mediated effector functions. Such functions comprise in the case of a helper T cell (CD4⁺ T cell) the release of cytokines such as Interleukin-2 and/or the activation of CD8⁺ lymphocytes (CTLs) and/or B-cells, and in the case of CTL the elimination of cells, i.e., cells characterized by expression of an antigen, for example, via apoptosis or perforin-mediated cell lysis, production of cytokines such as IFN- γ and TNF- α , and specific cytolytic killing of antigen expressing target cells.

The term "immunoreactive cell" or "immune effector cell" in the context of the present invention relates to a cell which exerts effector functions during an immune reaction. An "immunoreactive cell" preferably is capable of binding an antigen such as an antigen expressed on the surface of a cell and mediating an immune response. For example, such cells secrete cytokines and/or chemokines, kill microbes, secrete antibodies, recognize infected or cancerous cells, and optionally eliminate such cells. For example, immunoreactive cells comprise T cells (cytotoxic T cells, helper T cells, tumor infiltrating T cells), B cells, natural killer cells, neutrophils, macrophages, and dendritic cells. Preferably, in the context of the present invention, "immunoreactive cells" are T cells, preferably CD4⁺ and/or CD8⁺ T cells. According to the invention, the term "immunoreactive cell" also includes a cell which can mature into an immune cell (such as T cell, in particular T helper cell, or cytolytic T cell) with suitable stimulation. Immunoreactive cells comprise CD34⁺ hematopoietic stem cells, immature and mature T cells and immature and mature B cells. The differentiation of T cell precursors into a cytolytic T cell, when exposed to an antigen, is similar to clonal selection of the immune system.

Preferably, an "immunoreactive cell" or "immune effector cell" recognizes an antigen with some degree of specificity, in particular if present on the surface of antigen presenting cells or diseased cells such as cancer cells. Preferably, said recognition enables the cell that recognizes an antigen to be responsive or reactive. If the cell is a helper T cell ($CD4^+$ T cell) such responsiveness or reactivity may involve the release of cytokines and/or the activation of $CD8^+$ lymphocytes (CTLs) and/or B-cells. If the cell is a CTL such responsiveness or reactivity may involve the elimination of cells, i.e., cells characterized by expression of an antigen, for example, via apoptosis or perforin-mediated cell lysis. According to the invention, CTL responsiveness may include sustained calcium flux, cell division, production of cytokines such as $IFN-\gamma$ and $TNF-\alpha$, up-regulation of activation markers such as CD44 and CD69, and specific cytolytic killing of antigen expressing target cells. CTL responsiveness may also be determined using an artificial reporter that accurately indicates CTL responsiveness. Such CTL that recognizes an antigen and are responsive or reactive are also termed "antigen-responsive CTL" herein.

A "lymphoid cell" is a cell which, optionally after suitable modification, e.g. after transfer of a T cell receptor or antigen receptor, is capable of producing an immune response such as a cellular immune response, or a precursor cell of such cell, and includes lymphocytes, preferably T lymphocytes, lymphoblasts, and plasma cells. A lymphoid cell may be an immunoreactive cell or immune effector cell as described herein. A preferred lymphoid cell is a T cell which can be modified to express a T cell receptor or antigen receptor on the cell surface. In one embodiment, the lymphoid cell lacks endogenous expression of a T cell receptor.

The terms "T cell" and "T lymphocyte" are used interchangeably herein and include T helper cells ($CD4^+$ T cells) and cytotoxic T cells (CTLs, $CD8^+$ T cells) which comprise cytolytic T cells.

T cells belong to a group of white blood cells known as lymphocytes, and play a central role in cell-mediated immunity. They can be distinguished from other lymphocyte types, such as B cells and natural killer cells by the presence of a special receptor on their cell surface called T cell receptors (TCR). The thymus is the principal organ responsible for the maturation of T cells. Several different subsets of T cells have been discovered, each with a distinct function.

T helper cells assist other white blood cells in immunologic processes, including maturation of B cells into plasma cells and activation of cytotoxic T cells and macrophages, among other functions. These cells are also known as CD4⁺ T cells because they express the CD4 protein on their surface. Helper T cells become activated when they are presented with peptide antigens by MHC class II molecules that are expressed on the surface of antigen presenting cells (APCs). Once activated, they divide rapidly and secrete small proteins called cytokines that regulate or assist in the active immune response.

Cytotoxic T cells destroy virally infected cells and tumor cells, and are also implicated in transplant rejection. These cells are also known as CD8⁺ T cells since they express the CD8 glycoprotein at their surface. These cells recognize their targets by binding to antigen associated with MHC class I, which is present on the surface of nearly every cell of the body.

A majority of T cells have a T cell receptor (TCR) existing as a complex of several proteins. The actual T cell receptor is composed of two separate peptide chains, which are produced from the independent T cell receptor alpha and beta (TCR α and TCR β) genes and are called α - and β -TCR chains. $\gamma\delta$ T cells (gamma delta T cells) represent a small subset of T cells that possess a distinct T cell receptor (TCR) on their surface. However, in $\gamma\delta$ T cells, the TCR is made up of one γ -chain and one δ -chain. This group of T cells is much less common (2% of total T cells) than the $\alpha\beta$ T cells.

All T cells originate from hematopoietic stem cells in the bone marrow. Hematopoietic progenitors derived from hematopoietic stem cells populate the thymus and expand by cell division to generate a large population of immature thymocytes. The earliest thymocytes express neither CD4 nor CD8, and are therefore classed as double-negative (CD4-CD8-) cells. As they progress through their development they become double-positive thymocytes (CD4⁺CD8⁺), and finally mature to single-positive (CD4⁺CD8- or CD4-CD8⁺) thymocytes that are then released from the thymus to peripheral tissues.

T cells may generally be prepared *in vitro* or *ex vivo*, using standard procedures. For example, T cells may be isolated from bone marrow, peripheral blood or a fraction of bone marrow or peripheral blood of a mammal, such as a patient, using a commercially available cell separation system. Alternatively, T cells may be derived from related or unrelated humans, non-human

animals, cell lines or cultures. A sample comprising T cells may, for example, be peripheral blood mononuclear cells (PBMC).

The T cells to be used according to the invention may express an endogenous T cell receptor or may lack expression of an endogenous T cell receptor.

Nucleic acids such as RNA encoding an antigen receptor may be introduced into T cells or other cells with lytic potential, in particular lymphoid cells.

The term "antigen receptor targeted to an antigen" or similar terms relate to an antigen receptor which when present on an immune effector cell such as a T cell recognizes the antigen such as on the surface of antigen presenting cells or diseased cells such as cancer cells, such that the immune effector cell is stimulated, primed and/or expanded or exerts effector functions of immune effector cells as described above.

The term "antigen-specific T cell" or similar terms relate to a T cell which, in particular when provided with an antigen receptor, recognizes the antigen to which the antigen receptor is targeted such as on the surface of antigen presenting cells or diseased cells such as cancer cells and preferably exerts effector functions of T cells as described above. T cells and other lymphoid cells are considered to be specific for antigen if the cells kill target cells expressing an antigen. T cell specificity may be evaluated using any of a variety of standard techniques, for example, within a chromium release assay or proliferation assay. Alternatively, synthesis of lymphokines (such as interferon- γ) can be measured.

The term "major histocompatibility complex" and the abbreviation "MHC" include MHC class I and MHC class II molecules and relate to a complex of genes which occurs in all vertebrates. MHC proteins or molecules are important for signaling between lymphocytes and antigen presenting cells or diseased cells in immune reactions, wherein the MHC proteins or molecules bind peptides and present them for recognition by T cell receptors. The proteins encoded by the MHC are expressed on the surface of cells, and display both self antigens (peptide fragments from the cell itself) and nonself antigens (e.g., fragments of invading microorganisms) to a T cell.

According to the invention the term "antigen receptor" includes engineered receptors, which confer an arbitrary specificity such as the specificity of a monoclonal antibody onto an immune effector cell such as a T cell. In this way, a large number of antigen-specific T cells can be generated for adoptive cell transfer. Thus, an antigen receptor according to the invention may be present on T cells, e.g. instead of or in addition to the T cell's own T cell receptor. Such T cells do not necessarily require processing and presentation of an antigen for recognition of the target cell but rather may recognize preferably with specificity any antigen present on a target cell. Preferably, said antigen receptor is expressed on the surface of the cells. For the purpose of the present invention T cells comprising an antigen receptor are comprised by the term "T cell" as used herein. Specifically, according to the invention, the term "antigen receptor" includes artificial receptors comprising a single molecule or a complex of molecules which recognize, i.e. bind to, a target structure (e.g. an antigen) on a target cell such as a cancer cell (e.g. by binding of an antigen binding site or antigen binding domain to an antigen expressed on the surface of the target cell) and may confer specificity onto an immune effector cell such as a T cell expressing said antigen receptor on the cell surface. Preferably, recognition of the target structure by an antigen receptor results in activation of an immune effector cell expressing said antigen receptor. An antigen receptor may comprise one or more protein units said protein units comprising one or more domains as described herein. The term "antigen receptor" preferably does not include T cell receptors. According to the invention the term "antigen receptor" is preferably synonymous with the terms "chimeric antigen receptor (CAR)", "chimeric T cell receptor" and "artificial T cell receptor".

According to the invention, antigen can be recognized by an antigen receptor through any antigen recognition domains (herein also referred to simply as "domains") able to form an antigen binding site such as through antigen-binding portions of antibodies and T cell receptors which may reside on the same or different peptide chains. In one embodiment, the two domains forming an antigen binding site are derived from an immunoglobulin. In another embodiment, the two domains forming an antigen binding site are derived from a T cell receptor. Particularly preferred are antibody variable domains, such as single-chain variable fragments (scFv) derived from monoclonal antibodies and T cell receptor variable domains, in particular TCR alpha and beta single chains. In fact almost anything that binds a given target with high affinity can be used as an antigen recognition domain.

In one embodiment, an antigen receptor of the invention comprises at least four immunoglobulin variable domains forming at least two binding sites, wherein the two binding sites may bind to the same or different epitopes, which epitopes may be located on the same or different antigens. In one embodiment the antigen receptor comprises a variable domain (or region) of a heavy chain of an immunoglobulin (VH) with a specificity for a first epitope (VH(1)), a variable domain (or region) of a light chain of an immunoglobulin (VL) with a specificity for a first epitope (VL(1)), a variable domain (or region) of a heavy chain of an immunoglobulin (VH) with a specificity for a second epitope (VH(2)), and a variable domain (or region) of a light chain of an immunoglobulin (VL) with a specificity for a second epitope (VL(2)), which first and second epitopes may be the same or different and may be located on the same or different antigens. In one embodiment, VH(1) is able to interact and form an antigen binding site with VL(1) and VH(2) is able to interact and form an antigen binding site with VL(2), while VH(1) is not able to interact and form an antigen binding site with VL(2) and VH(2) is not able to interact and form an antigen binding site with VL(1). In another embodiment, however, VH(1) is able to interact and form an antigen binding site with VL(1) as well as VL(2) and VH(2) is able to interact and form an antigen binding site with VL(2) as well as VL(1). In the latter embodiment, VH(1) and VH(2) may be identical or at least derived from the same immunoglobulin and VL(1) and VL(2) may be identical or at least derived from the same immunoglobulin.

In one aspect, the invention relates to an antigen receptor, also termed combinatory antigen receptor herein, which receptor comprises a first peptide chain and a second peptide chain, wherein the first peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; the second peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain; wherein the first domain from the first peptide chain forms together with one of the domains from the second peptide chain a first antigen binding site, and wherein the second domain from the first peptide chain forms together with the other domain from the second peptide chain a second antigen binding site.

In one embodiment, the combinatory antigen receptor of the invention comprises a heavy chain variable domain connected to a light chain variable domain on each of both peptide chains wherein formation of two antigen binding sites takes place through interaction between a heavy chain variable domain and a light chain variable domain on different peptide chains. In one embodiment, the combinatory antigen receptor of the invention comprises two peptide chains,

wherein one peptide chain comprises VL(1) and VH(2) and the other polypeptide chain comprises VH(1) and VL(2). In another embodiment, the combinatory antigen receptor of the invention comprises a heavy chain variable domain connected to a heavy chain variable domain on one peptide chain and a light chain variable domain connected to a light chain variable domain on the other peptide chain wherein formation of two antigen binding sites takes place through interaction between a heavy chain variable domain and a light chain variable domain on different peptide chains. In one embodiment, the combinatory antigen receptor of the invention comprises two peptide chains, wherein one peptide chain comprises VH(1) and VH(2) and the other peptide chain comprises VL(1) and VL(2).

In one embodiment, the combinatory antigen receptor of the invention comprises a first peptide chain wherein the heavy chain variable region (VH) and the light chain variable region (VL) are preferably arranged, from N-terminus to C-terminus, in the order VH(1)-VL(2) and a second peptide chain wherein the heavy chain variable region (VH) and the light chain variable region (VL) are preferably arranged, from N-terminus to C-terminus, in the order VL(1)-VH(2). The immunoreceptor signal transmission domain is preferably located C-terminal to the arrangement of variable regions and preferably comprises a constant region of a T cell receptor alpha chain or a portion thereof located on one of the peptide chains and a constant region of a T cell receptor beta chain located on the other of the peptide chains.

In one embodiment, the combinatory antigen receptor of the invention comprises a first peptide chain wherein the heavy chain variable region (VH) and the light chain variable region (VL) are preferably arranged, from N-terminus to C-terminus, in the order VH(1)-VH(2) and a second peptide chain wherein the heavy chain variable region (VH) and the light chain variable region (VL) are preferably arranged, from N-terminus to C-terminus, in the order VL(1)-VL(2). The immunoreceptor signal transmission domain is preferably located C-terminal to the arrangement of variable regions and preferably comprises a constant region of a T cell receptor alpha chain or a portion thereof located on one of the peptide chains and a constant region of a T cell receptor beta chain or a portion thereof located on the other of the peptide chains.

In one aspect, the invention relates to an antigen receptor, also termed tandem antigen receptor herein, which receptor comprises a first peptide chain and a second peptide chain, wherein the first peptide chain comprises at least four domains, and an immunoreceptor signal transmission domain; the second peptide chain comprises an immunoreceptor signal transmission domain;

wherein two of the domains from the first peptide chain form a first antigen binding site, and wherein the other two domains from the first peptide chain form a second antigen binding site.

In one embodiment, the tandem antigen receptor of the invention comprises a heavy chain variable domain connected to a light chain variable domain connected to a further heavy chain variable domain connected to a light chain variable domain on the first peptide chain wherein formation of two antigen binding sites takes place through interaction between a heavy chain variable domain and a light chain variable domain on the same peptide chain. Thus, the first peptide chain comprises VH(1) and VL(1) as well as VH(2) and VL(2) on the same peptide chain. In one embodiment, the tandem antigen receptor of the invention may be considered to comprise two scFv molecules connected via a linker peptide on the first peptide chain.

In one embodiment, the tandem antigen receptor of the invention comprises a first peptide chain wherein the heavy chain variable region (VH) and the light chain variable region (VL) are preferably arranged, from N-terminus to C-terminus, in the order VH(1)-VL(1)-VH(2)-VL(2), VL(1)-VH(1)-VH(2)-VL(2), VH(1)-VL(1)-VL(2)-VH(2), or VL(1)-VH(1)-VL(2)-VH(2). The immunoreceptor signal transmission domain is preferably located C-terminal to the arrangement of variable regions and preferably comprises a constant region of a T cell receptor alpha chain or a portion thereof or a constant region of a T cell receptor beta chain or a portion thereof. The immunoreceptor signal transmission domain located on the second peptide chain preferably comprises a constant region of a T cell receptor alpha chain or a portion thereof if the first peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof or a constant region of a T cell receptor beta chain or a portion thereof if the first peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof.

Antigen receptors of the invention have at least two antigen binding sites and thus, are at least bivalent. As noted above, the binding sites of the antigen receptors of the invention may bind to the same or different epitopes, which epitopes may be located on the same or different antigens. If the binding sites bind to the same epitopes, in particular on the same antigen, the two binding sites may be identical or essentially identical and/or may be formed by identical or essentially identical domains, wherein such identical or essentially identical domains may be derived, for example, from the same immunoglobulin. If the binding sites bind to different epitopes, either on the same or different antigens, the two binding sites are different and are formed by different domains, wherein such different domains may be derived from different immunoglobulins. In the

case of such different domains it is preferred that the domains having different epitope specificities do not interact or do not substantially interact with each other, i.e. VH(1) is not able to interact and form an antigen binding site with VL(2) and VH(2) is not able to interact and form an antigen binding site with VL(1). Consequently, VH(1) interacts and forms an antigen binding site with VL(1) and VH(2) interact and forms an antigen binding site with VL(2). If a combinatory antigen receptor of the invention comprises two peptide chains, wherein one peptide chain comprises VH(1) and VL(2) and the other peptide chain comprises VH(2) and VL(1), this results in the peptide chains not being able to form antigen binding sites through intramolecular interaction of domains.

The two domains of an antigen receptor of the invention forming an antigen binding site also can be derived from a T cell receptor and can be fragments or portions thereof that maintain antigen-specific binding, in particular binding to the peptide-MHC complex, such as the variable regions of a T cell receptor.

According to the invention, the term "variable region of a T cell receptor" relates to the variable domains of the TCR chains. The variable region of both the TCR α -chain and β -chain have three hypervariable or complementarity determining regions (CDRs), whereas the variable region of the β -chain has an additional area of hypervariability (HV4) that does not normally contact antigen and therefore is not considered a CDR. CDR3 is the main CDR responsible for recognizing processed antigen, although CDR1 of the α -chain has also been shown to interact with the N-terminal part of the antigenic peptide, whereas CDR1 of the β -chain interacts with the C-terminal part of the peptide. CDR2 is thought to recognize the MHC. CDR4 of the β -chain is not thought to participate in antigen recognition, but has been shown to interact with superantigens.

The above disclosure relating to immunoglobulin variable domains applies in a corresponding manner to T cell receptor variable domains. An antigen receptor of the invention instead of a variable domain of a heavy chain of an immunoglobulin (VH) with a specificity for a first epitope (VH(1)) and a variable domain of a light chain of an immunoglobulin (VL) with a specificity for a first epitope (VL(1)) may comprise a variable domain of a TCR α -chain of a TCR with a specificity for a first epitope and a variable domain of a TCR β -chain of a TCR with a specificity for a first epitope. Alternatively or additionally, an antigen receptor of the invention instead of a variable domain of a heavy chain of an immunoglobulin (VH) with a specificity for a

second epitope (VH(2)), and a variable domain of a light chain of an immunoglobulin (VL) with a specificity for a second epitope (VL(2)) may comprise a variable domain of a TCR α -chain of a TCR with a specificity for a second epitope and a variable domain of a TCR β -chain of a TCR with a specificity for a second epitope.

Since each antigen binding site is formed from two domains, each domain can comprise a portion or a fragment of an immunoglobulin or T cell receptor, respectively. The individual portion or fragment alone may not be able to bind the antigen but when the two individual portions or fragments associate they together form or recreate the antigen binding structure of the original immunoglobulin or T cell receptor and, thus, are able to bind the same antigen, preferably with the same affinity.

Following antigen recognition, receptors preferably cluster and a signal is transmitted to the cell. In this respect, an "immunoreceptor signal transmission domain" or "T cell signaling domain" is a domain which is involved in transmitting an activation signal to the T cell after antigen is bound. Such signal transmission may be enabled by the antigen receptors of the invention comprising a constant or invariant region of a T cell receptor chain or a constant or invariant region of an immune cell Fc receptor chain or a portion of the constant or invariant region, such as a constant region of a T cell receptor alpha chain or a portion thereof, on one peptide chain and comprising the corresponding constant or invariant region of a T cell receptor chain or corresponding constant or invariant region of an immune cell Fc receptor chain or a portion of the constant or invariant region, such as a constant region of a T cell receptor beta chain or a portion thereof, on the other peptide chain. In this respect, the CD3 complex denotes an antigen that is expressed on mature human T cells, thymocytes and a subset of natural killer cells as part of the multimolecular T cell receptor (TCR) complex. The T cell co-receptor is a protein complex and is composed of four distinct chains. In mammals, the complex contains a CD3 γ chain, a CD3 δ chain, and two CD3 ϵ chains. These chains associate with the T cell receptor (TCR) and the ζ -chain to generate an activation signal in T lymphocytes. The TCR, ζ -chain, and CD3 molecules together comprise the TCR complex. CD3 is responsible for the signal transduction of the TCR. As described by Lin and Weiss, Journal of Cell Science 114, 243-244 (2001), activation of the TCR complex by binding of MHC-presented specific antigen epitopes results in the phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by Src family kinases, triggering recruitment of further kinases which results in T cell activation including Ca²⁺ release. Clustering of CD3 on T cells, e.g. by immobilized anti-CD3-antibodies,

leads to T cell activation similar to the engagement of the T cell receptor, but independent from its clone typical specificity.

The antigen receptor signal transmission domain preferably at a minimum serves to interact with the native cellular signal transduction complex, e.g., the CD3 complex, which is responsible for transmitting the signal of antigen binding to an antigen receptor into the cell, resulting in immune cell activation. The identity of the signal transmission domain is limited only in that it has the ability to interact with the native signal transduction complex to induce activation of the immune cell upon binding of the antigen to the antigen receptor.

Preferably, the signal transmission domain on one peptide chain will form a dimer with the signal transmission domain on the second chain, for example, through disulfide bridges. Preferred signal transmission domains can comprise a constant or invariant region of a T cell receptor chain or a constant or invariant region of an immune cell Fc receptor chain or a portion of the constant or invariant region. Preferred signal transmission domains can comprise the constant region of the alpha, beta, gamma or delta chains of a T cell receptor or portion thereof, as well as the D2 or D3 invariant regions of the constant domain of an immune cell Fc receptor or a portion thereof. In a preferred embodiment, the first peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof, or the first peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof. In another embodiment, the first peptide chain comprises a constant region of a T cell receptor gamma chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor delta chain or a portion thereof, or the first peptide chain comprises a constant region of a T cell receptor delta chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor gamma chain or a portion thereof. Optionally, the signal transmission domains can be modified such that additional disulfide bonds between the chains are created, leading to more effective dimer formation and to greater stability of the dimer.

While not wanting to be limited to a particular mechanism of action, it is believed that the two peptide chains of the antigen receptor of the invention, when expressed on the surface of an immune cell, form a dimer due to interactions (e.g., disulfide bonding) at least between the

individual immunoreceptor signal transmission domains on the two chains, as well as form a complex with the endogenous CD3 complex involved in physiological T cell receptor signal transduction. However, the invention may also include the direct fusion to CD3 ζ or any other immune cell signaling domain (CD3, CD3 subunit Fc γ R) instead of TCR C α and C β -domains. Upon antigen binding, it is believed that a signal is transmitted into the cell leading to the activation of the immune cell and to the generation of an antigen-specific immune response. Further, it is believed that inter-chain antigen binding provides for a more stable antigen-antigen receptor-endogenous CD3 signal transduction module, which greater stability in turn allows for a more effective stimulation of an antigen-specific immune response, as compared to monovalent receptors and bivalent receptors only capable of intra-chain antigen binding. This greater stability also is believed to allow for the option of using only human origin immunoreceptor signal transmission domains (*e.g.*, minimal or no substitution of a human-derived amino acid sequence with an amino acid sequence derived from another species, such as mouse). Thus, any potential unwanted immune response against the antigen receptor itself can be avoided.

Antigen receptors according to the invention or peptide chains thereof may in addition to the domains forming the antigen binding sites and immunoreceptor signal transmission domains including CD3 ζ or any other immune cell signaling domain also comprise one or more co-stimulation domains. The co-stimulation domains serve to enhance the proliferation and survival of the T cells such as cytotoxic T cells upon binding of the antigen receptor to a targeted moiety. The identity of the co-stimulation domains is limited only in that they have the ability to enhance cellular proliferation and survival upon binding of the targeted moiety by the antigen receptor. Suitable co-stimulation domains include CD28, CD137 (4-1BB), a member of the tumor necrosis factor (TNF) receptor family, CD134 (OX40), a member of the TNFR-superfamily of receptors, and CD278 (ICOS), a CD28-superfamily co-stimulatory molecule expressed on activated T cells. The skilled person will understand that sequence variants of these noted co-stimulation domains can be used without adversely impacting the invention, where the variants have the same or similar activity as the domain on which they are modeled. Such variants will have at least about 80% sequence identity to the amino acid sequence of the domain from which they are derived. In some embodiments of the invention, the antigen receptor constructs or peptide chains thereof comprise two co-stimulation domains. While the particular combinations include all possible variations of the four noted domains, specific examples include CD28+CD137 (4-1BB) and CD28+CD134 (OX40).

The antigen receptors of the present invention or peptide chains thereof may comprise one or more co-stimulation domains, and immunoreceptor signal transmission domains, linked in a N-terminal to C-terminal direction. However, the antigen receptors of the present invention or peptide chains thereof are not limited to this arrangement and other arrangements are acceptable and include immunoreceptor signal transmission domains, and one or more co-stimulation domains.

It will be understood that because the domains forming the antigen binding sites must be free to bind antigen, the placement of these domains in the fusion protein will generally be such that display of the region on the exterior of the cell is achieved. In the same manner, because the co-stimulation domains, and immunoreceptor signal transmission domains serve to induce activity and proliferation of the T cells, the fusion protein will generally display these domains in the interior of the cell. The antigen receptors may include additional elements, such as a signal peptide to ensure proper export of the fusion protein to the cells surface, a transmembrane domain to ensure the fusion protein is maintained as an integral membrane protein, and a hinge domain (or spacer region) that imparts flexibility to the domains forming the antigen binding sites and allows strong binding to antigen.

Optionally, the antigen receptors of the invention can further comprise a linker, which linker can be an arbitrary amino acid sequence or other chemical compound useful as a spacer between amino acid sequences. The linker is generally designed to provide for flexibility and protease resistance. For example, the linker can be between the first and second domains on the first peptide chain and/or between the first and second domains on the second peptide chain of a combinatory antigen receptor of the invention. In another embodiment, the linker can be between any two of the at least four domains on the first peptide chain, i.e., between the first and second domains and/or between the second and third domains and/or between the third and fourth domains of a tandem antigen receptor of the invention. Optionally, the linker can be present between the domains that form the antigen binding sites and the immunoreceptor signal transmission domain. Any type of linker known in the art that allows the domains to form an antigen binding site or does not interfere with antigen binding is encompassed by the invention. In specific embodiments, the linker can be an arbitrary amino acid sequence and can be at least 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or at least 100 amino acid residues in length. An amino acid linker is typically rich in glycine for flexibility, as well as serine and threonine for solubility. In one embodiment, the linker is one or more (1, 2, 3, 4, 5, 6,

7, 8 or 9) repeats of four glycine residues followed by a serine residue (Gly4Ser). In certain embodiments, the linker can be a hinge region of an antibody or a fragment thereof.

The antigen receptor of the invention can further comprise another domain anchoring the antigen receptor on the membrane, such as a classical transmembrane domain. Preferably, the transmembrane domain is incorporated in or is a part of the signal transmission domain.

In other embodiments, antigen receptors or peptide chains of antigen receptors of the invention can further comprise other domains, such as additional domains involved in or enhancing antigen binding, signal sequences for membrane bound expression or for secretion, domains that provide improved dimerization, and a transmembrane domain, when not already a part of the immunoreceptor signal transmission domain. In certain embodiments, the transmembrane domain can be a hydrophobic alpha helix that spans the membrane.

Preferably, a signal sequence or signal peptide is a sequence or peptide that allows for sufficient passage through the secretory pathway and expression on the cell surface such that an antigen receptor, for example, may bind an antigen present in the extracellular environment. Preferably, the signal sequence or signal peptide is cleavable and is removed from the mature peptide chains. The signal sequence or signal peptide preferably is chosen with respect to the cell or organism wherein the peptide chains are produced in.

In a particular embodiment, a peptide chain of a combinatory antigen receptor of the invention can comprise the structure: NH₂-signal peptide-first domain involved in antigen binding-optional linker-second domain involved in antigen binding-optional linker-immunoreceptor signal transmission domain-COOH.

In a particular embodiment, a first peptide chain of a tandem antigen receptor of the invention can comprise the structure: NH₂-signal peptide-first domain involved in antigen binding-optional linker-second domain involved in antigen binding-optional linker-third domain involved in antigen binding-optional linker-fourth domain involved in antigen binding-optional linker-immunoreceptor signal transmission domain-COOH.

With respect to tandem antigen receptors of the invention, the first and second (from N-terminus to C-terminus) domains involved in the formation of an antigen binding site of a first peptide

chain are also termed "N-terminal domains" and the third and fourth (from N-terminus to C-terminus) domains involved in the formation of an antigen binding site of a first peptide chain are also termed "C-terminal domains" herein.

Exemplary antigen receptors of the invention, include but are not limited to those formed by the first and second peptide chains having the structures listed in the Table I below (VH being the variable region of a heavy chain of an immunoglobulin or a portion thereof; VL being the variable region of a light chain of an immunoglobulin or portion thereof; C1 and C2 being the immunoreceptor signal transmission domains that will form a dimer with each other, e.g., the constant or invariant region of an immune cell Fc receptor chain, or the constant or invariant region of a T cell receptor chain, or a portion of the constant or invariant region):

Table I

First peptide chain	Second peptide chain
VH(1)-VL(2)-C1	VL(1)-VH(2)-C2
VH(1)-VH(2)-C1	VL(1)-VL(2)-C2
VH(1)-VH(2)-C1	VL(2)-VL(1)-C2
VH(1)-VL(2)-C1	VH(2)-VL(1)-C2
VH(1)-VL(1)-VH(2)-VL(2)-C1	C2
VL(1)-VH(1)-VH(2)-VL(2)-C1	C2
VH(1)-VL(1)-VL(2)-VH(2)-C1	C2
VL(1)-VH(1)-VL(2)-VH(2)-C1	C2

As defined above, the antigen receptor comprises a variable domain of a heavy chain of an immunoglobulin (VH) with a specificity for a first epitope (VH(1)), a variable domain of a light chain of an immunoglobulin (VL) with a specificity for a first epitope (VL(1)), a variable domain of a heavy chain of an immunoglobulin (VH) with a specificity for a second epitope (VH(2)), and a variable domain of a light chain of an immunoglobulin (VL) with a specificity for a second epitope (VL(2)), which first and second epitopes may be the same or different and may be located on the same or different antigens. In one embodiment, VH(1) is able to interact and form an antigen binding site with VL(1) and VH(2) is able to interact and form an antigen binding site with VL(2), while VH(1) is not able to interact and form an antigen binding site with VL(2) and VH(2) is not able to interact and form an antigen binding site with VL(1). In another embodiment, however, VH(1) is able to interact and form an antigen binding site with VL(1) as

well as VL(2) and VH(2) is able to interact and form an antigen binding site with VL(2) as well as VL(1). In the latter embodiment, VH(1) and VH(2) may be identical or at least derived from the same immunoglobulin and VL(1) and VL(2) may be identical or at least derived from the same immunoglobulin.

In specific embodiments, the C1 and C2 domains of the first and second peptide chains listed in Table 1 are the constant regions of the T cell receptor alpha and beta chains, respectively, or a portion thereof.

In a preferred embodiment, when the two domains on one peptide chain are both an immunoglobulin heavy chain variable region or a portion thereof and the two domains on the other chain are both an immunoglobulin light chain variable region or a portion thereof, a linker is present between the first and second domains on both peptide chains. The linker can be an arbitrary amino acid sequence between 10 and 25 amino acids in length, more preferably 15 amino acids in length. In a specific embodiment, the linker is 3 repeats of the 5-mer amino acid sequence (Gly4Ser).

In certain embodiments of the invention, the amino acid sequences of the first and second peptide chains, such as those comprising one or more of the domains that form the antigen binding sites or the immunoreceptor signal transmission domain, are of mammalian origin, preferably mouse origin, and more preferably human origin. In one embodiment, the amino acid sequences are of human origin but have been murinized by the substitution of one or more amino acids in the human sequence with the amino acid found in the corresponding position in the mouse sequence. Such substitution can provide for greater dimerization or stability or ability to transmit a signal into the cell upon antigen binding. In yet another embodiment, the amino acid sequences are of mouse origin and have been humanized.

According to the invention an antigen receptor may replace the function of a T cell receptor as described above and, in particular, may confer reactivity such as cytolytic activity to a cell such as a T cell as described above. However, in contrast to the binding of the T cell receptor to an antigen peptide-MHC complex as described above, an antigen receptor may in certain embodiments bind to an antigen, in particular when expressed on the cell surface.

The amino acid sequences of the peptide chains, including any of the domains or linkers, can be modified. For example, and as is appreciated by those skilled in the art, the sequences of the variable regions of antibodies and T cell receptors can be modified without losing the ability to bind to a target and consequently the amino acid sequence of the antigen binding sites can be similarly modified without losing the ability to bind a target. For example, the amino acid sequence of a domain forming an antigen binding site can be identical or highly homologous to the variable region of the antibody from which it was derived. By "highly homologous" it is contemplated that from 1 to 5, preferably from 1 to 4, such as 1 to 3 or 1 or 2 substitutions may be made. In one embodiment, a peptide chain may include natural amino acids and non-natural amino acids. In another embodiment, a peptide chain merely includes natural amino acids. The term "non-natural amino acid" refers to an amino acid having a structure different from those of the 20 natural amino acid species. Since non-natural amino acids have structures similar to those of natural amino acids, non-natural amino acids may be classified as derivatives or analogs of given natural amino acids.

The present invention also encompasses derivatives of the antigen receptors and peptide chains described herein. According to the invention, "derivatives" are modified forms of proteins and peptides. Such modifications include any chemical modification and comprise single or multiple substitutions, deletions and/or additions of any molecules associated with the antigen receptor or peptide chain, such as carbohydrates, lipids and/or proteins or peptides. In one embodiment, "derivatives" of proteins or peptides include those modified analogs resulting from glycosylation, acetylation, phosphorylation, amidation, palmitoylation, myristoylation, isoprenylation, lipidation, alkylation, derivatization, introduction of protective/blocking groups, proteolytic cleavage or binding to an antigen. The term "derivative" also extends to all functional chemical equivalents of said antigen receptors and peptide chains. Preferably, a modified antigen receptor or peptide chain thereof has increased binding or dimerization ability and/or increased immune activating ability.

The cells used in connection with the antigen receptor system of the present invention are preferably T cells, in particular cytotoxic lymphocytes, preferably selected from cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. Upon activation, each of these cytotoxic lymphocytes triggers the destruction of target cells. For example, cytotoxic T cells trigger the destruction of target cells by either or both of the following means. First, upon activation T cells release cytotoxins such as perforin, granzymes, and granulysin.

Perforin and granzysin create pores in the target cell, and granzymes enter the cell and trigger a caspase cascade in the cytoplasm that induces apoptosis (programmed cell death) of the cell. Second, apoptosis can be induced via Fas-Fas ligand interaction between the T cells and target cells. The cytotoxic lymphocytes will preferably be autologous cells, although heterologous cells or allogenic cells can be used.

The term "immunoglobulin" relates to proteins of the immunoglobulin superfamily, preferably to antigen receptors such as antibodies or the B cell receptor (BCR). The immunoglobulins are characterized by a structural domain, i.e., the immunoglobulin domain, having a characteristic immunoglobulin (Ig) fold. The term encompasses membrane bound immunoglobulins as well as soluble immunoglobulins. Membrane bound immunoglobulins are also termed surface immunoglobulins or membrane immunoglobulins, which are generally part of the BCR. Soluble immunoglobulins are generally termed antibodies. Immunoglobulins generally comprise several chains, typically two identical heavy chains and two identical light chains which are linked via disulfide bonds. These chains are primarily composed of immunoglobulin domains, such as the V_L (variable light chain) domain, C_L (constant light chain) domain, and the C_H (constant heavy chain) domains C_{H1} , C_{H2} , C_{H3} , and C_{H4} . There are five types of mammalian immunoglobulin heavy chains, i.e., α , δ , ϵ , γ , and μ which account for the different classes of antibodies, i.e., IgA, IgD, IgE, IgG, and IgM. As opposed to the heavy chains of soluble immunoglobulins, the heavy chains of membrane or surface immunoglobulins comprise a transmembrane domain and a short cytoplasmic domain at their carboxy-terminus. In mammals there are two types of light chains, i.e., lambda and kappa. The immunoglobulin chains comprise a variable region and a constant region. The constant region is essentially conserved within the different isotypes of the immunoglobulins, wherein the variable part is highly divers and accounts for antigen recognition.

The term "antibody" refers to a glycoprotein comprising at least two heavy (H) chains and two light (L) chains inter-connected by disulfide bonds. The term "antibody" includes monoclonal antibodies, recombinant antibodies, human antibodies, humanized antibodies and chimeric antibodies. Each heavy chain is comprised of a heavy chain variable region (abbreviated herein as V_H) and a heavy chain constant region. Each light chain is comprised of a light chain variable region (abbreviated herein as V_L) and a light chain constant region. The V_H and V_L regions can be further subdivided into regions of hypervariability, termed complementarity determining regions (CDR), interspersed with regions that are more conserved, termed framework regions

(FR). Each VH and VL is composed of three CDRs and four FRs, arranged from amino-terminus to carboxy-terminus in the following order: FR1, CDR1, FR2, CDR2, FR3, CDR3, FR4. The variable regions of the heavy and light chains contain a binding domain that interacts with an antigen. The constant regions of the antibodies may mediate the binding of the immunoglobulin to host tissues or factors, including various cells of the immune system (e.g., effector cells) and the first component (C1q) of the classical complement system.

The term "monoclonal antibody" as used herein refers to a preparation of antibody molecules of single molecular composition. A monoclonal antibody displays a single binding specificity and affinity. In one embodiment, the monoclonal antibodies are produced by a hybridoma which includes a B cell obtained from a non-human animal, e.g., mouse, fused to an immortalized cell.

The term "recombinant antibody", as used herein, includes all antibodies that are prepared, expressed, created or isolated by recombinant means, such as (a) antibodies isolated from an animal (e.g., a mouse) that is transgenic or transchromosomal with respect to the immunoglobulin genes or a hybridoma prepared therefrom, (b) antibodies isolated from a host cell transformed to express the antibody, e.g., from a transfectoma, (c) antibodies isolated from a recombinant, combinatorial antibody library, and (d) antibodies prepared, expressed, created or isolated by any other means that involve splicing of immunoglobulin gene sequences to other DNA sequences.

The term "human antibody", as used herein, is intended to include antibodies having variable and constant regions derived from human germline immunoglobulin sequences. Human antibodies may include amino acid residues not encoded by human germline immunoglobulin sequences (e.g., mutations introduced by random or site-specific mutagenesis *in vitro* or by somatic mutation *in vivo*).

The term "humanized antibody" refers to a molecule having an antigen binding site that is substantially derived from an immunoglobulin from a non-human species, wherein the remaining immunoglobulin structure of the molecule is based upon the structure and/or sequence of a human immunoglobulin. The antigen binding site may either comprise complete variable domains fused onto constant domains or only the complementarity determining regions (CDR) grafted onto appropriate framework regions in the variable domains. Antigen binding sites may be wild-type or modified by one or more amino acid substitutions, e.g. modified to resemble

human immunoglobulins more closely. Some forms of humanized antibodies preserve all CDR sequences (for example a humanized mouse antibody which contains all six CDRs from the mouse antibody). Other forms have one or more CDRs which are altered with respect to the original antibody.

The term "chimeric antibody" refers to those antibodies wherein one portion of each of the amino acid sequences of heavy and light chains is homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular class, while the remaining segment of the chain is homologous to corresponding sequences in another. Typically the variable region of both light and heavy chains mimics the variable regions of antibodies derived from one species of mammals, while the constant portions are homologous to sequences of antibodies derived from another. One clear advantage to such chimeric forms is that the variable region can conveniently be derived from presently known sources using readily available B-cells or hybridomas from non-human host organisms in combination with constant regions derived from, for example, human cell preparations. While the variable region has the advantage of ease of preparation and the specificity is not affected by the source, the constant region being human, is less likely to elicit an immune response from a human subject when the antibodies are injected than would the constant region from a non human source. However the definition is not limited to this particular example.

Antibodies may be derived from different species, including but not limited to mouse, rat, rabbit, guinea pig and human.

Antibodies described herein include IgA such as IgA1 or IgA2, IgG1, IgG2, IgG3, IgG4, IgE, IgM, and IgD antibodies. In various embodiments, the antibody is an IgG1 antibody, more particularly an IgG1, kappa or IgG1, lambda isotype (i.e. IgG1, κ , λ), an IgG2a antibody (e.g. IgG2a, κ , λ), an IgG2b antibody (e.g. IgG2b, κ , λ), an IgG3 antibody (e.g. IgG3, κ , λ) or an IgG4 antibody (e.g. IgG4, κ , λ).

The antigen receptors described herein may comprise antigen-binding portions of one or more antibodies. The terms "antigen-binding portion" of an antibody (or simply "binding portion") or "antigen-binding fragment" of an antibody (or simply "binding fragment") or similar terms refer to one or more fragments of an antibody that retain the ability to specifically bind to an antigen. It has been shown that the antigen-binding function of an antibody can be performed by

fragments of a full-length antibody. Examples of binding fragments encompassed within the term "antigen-binding portion" of an antibody include (i) Fab fragments, monovalent fragments consisting of the VL, VH, CL and CH domains; (ii) F(ab')₂ fragments, bivalent fragments comprising two Fab fragments linked by a disulfide bridge at the hinge region; (iii) Fd fragments consisting of the VH and CH domains; (iv) Fv fragments consisting of the VL and VH domains of a single arm of an antibody, (v) dAb fragments (Ward et al., (1989) Nature 341: 544-546), which consist of a VH domain; (vi) isolated complementarity determining regions (CDR), and (vii) combinations of two or more isolated CDRs which may optionally be joined by a synthetic linker. Furthermore, although the two domains of the Fv fragment, VL and VH, are coded for by separate genes, they can be joined, using recombinant methods, by a synthetic linker that enables them to be made as a single protein chain in which the VL and VH regions pair to form monovalent molecules (known as single chain Fv (scFv); see e.g., Bird et al. (1988) Science 242: 423-426; and Huston et al. (1988) Proc. Natl. Acad. Sci. USA 85: 5879-5883). Such single chain antibodies are also intended to be encompassed within the term "antigen-binding fragment" of an antibody. A further example is binding-domain immunoglobulin fusion proteins comprising (i) a binding domain polypeptide that is fused to an immunoglobulin hinge region polypeptide, (ii) an immunoglobulin heavy chain CH₂ constant region fused to the hinge region, and (iii) an immunoglobulin heavy chain CH₃ constant region fused to the CH₂ constant region. The binding domain polypeptide can be a heavy chain variable region or a light chain variable region. The binding-domain immunoglobulin fusion proteins are further disclosed in US 2003/0118592 and US 2003/0133939. These antibody fragments are obtained using conventional techniques known to those with skill in the art, and the fragments are screened for utility in the same manner as are intact antibodies.

A single-chain variable fragment (scFv) is a fusion protein of the variable regions of the heavy (VH) and light chains (VL) of immunoglobulins, connected with a linker peptide. The linker can either connect the N-terminus of the VH with the C-terminus of the VL, or vice versa. Divalent (or bivalent) single-chain variable fragments (di-scFvs, bi-scFvs) can be engineered by linking two scFvs. This can be done by producing a single peptide chain with two VH and two VL regions, yielding tandem scFvs.

The term "binding domain" or simply "domain" characterizes in connection with the present invention a structure, e.g. of an antibody, which binds to/interacts with a given target

structure/antigen/epitope, optionally when interacting with another domain. Thus, these domains according to the invention designate an "antigen binding site".

Antibodies and derivatives of antibodies are useful for providing binding domains such as antibody fragments, in particular for providing VL and VH regions.

Binding domains for an antigen which may be present within an antigen receptor have the ability of binding to (targeting) an antigen, i.e. the ability of binding to (targeting) an epitope present in an antigen, preferably an epitope located within the extracellular domain of an antigen. Preferably, binding domains for an antigen are specific for the antigen. Preferably, binding domains for an antigen bind to the antigen expressed on the cell surface. In particular preferred embodiments, binding domains for an antigen bind to native epitopes of an antigen present on the surface of living cells.

All antibodies and derivatives of antibodies such as antibody fragments as described herein for the purposes of the invention are encompassed by the term "antibody".

Antibodies can be produced by a variety of techniques, including conventional monoclonal antibody methodology, e.g., the standard somatic cell hybridization technique of Kohler and Milstein, *Nature* 256: 495 (1975). Although somatic cell hybridization procedures are preferred, in principle, other techniques for producing monoclonal antibodies can be employed, e.g., viral or oncogenic transformation of B-lymphocytes or phage display techniques using libraries of antibody genes.

The preferred animal system for preparing hybridomas that secrete monoclonal antibodies is the murine system. Hybridoma production in the mouse is a very well established procedure. Immunization protocols and techniques for isolation of immunized splenocytes for fusion are known in the art. Fusion partners (e.g., murine myeloma cells) and fusion procedures are also known.

Other preferred animal systems for preparing hybridomas that secrete monoclonal antibodies are the rat and the rabbit system (e.g. described in Spieker-Polet et al., *Proc. Natl. Acad. Sci. U.S.A.* 92:9348 (1995), see also Rossi et al., *Am. J. Clin. Pathol.* 124: 295 (2005)).

To generate antibodies, mice can be immunized with carrier-conjugated peptides derived from the antigen sequence, i.e. the sequence against which the antibodies are to be directed, an enriched preparation of recombinantly expressed antigen or fragments thereof and/or cells expressing the antigen, as described. Alternatively, mice can be immunized with DNA encoding the antigen or fragments thereof. In the event that immunizations using a purified or enriched preparation of the antigen do not result in antibodies, mice can also be immunized with cells expressing the antigen, e.g., a cell line, to promote immune responses.

The immune response can be monitored over the course of the immunization protocol with plasma and serum samples being obtained by tail vein or retroorbital bleeds. Mice with sufficient titers of immunoglobulin can be used for fusions. Mice can be boosted intraperitoneally or intravenously with antigen expressing cells 3 days before sacrifice and removal of the spleen to increase the rate of specific antibody secreting hybridomas.

To generate hybridomas producing monoclonal antibodies, splenocytes and lymph node cells from immunized mice can be isolated and fused to an appropriate immortalized cell line, such as a mouse myeloma cell line. The resulting hybridomas can then be screened for the production of antigen-specific antibodies. Individual wells can then be screened by ELISA for antibody secreting hybridomas. By Immunofluorescence and FACS analysis using antigen expressing cells, antibodies with specificity for the antigen can be identified. The antibody secreting hybridomas can be replated, screened again, and if still positive for monoclonal antibodies can be subcloned by limiting dilution. The stable subclones can then be cultured *in vitro* to generate antibody in tissue culture medium for characterization.

The ability of antibodies and other binding agents to bind an antigen can be determined using standard binding assays (e.g., ELISA, Western Blot, Immunofluorescence and flow cytometric analysis).

The term "binding" according to the invention preferably relates to a specific binding.

According to the present invention, an agent such as an antigen receptor is capable of binding to (targeting) a predetermined target if it has a significant affinity for said predetermined target and binds to said predetermined target in standard assays. "Affinity" or "binding affinity" is often measured by equilibrium dissociation constant (K_D). Preferably, the term "significant affinity"

refers to the binding to a predetermined target with a dissociation constant (K_D) of 10^{-5} M or lower, 10^{-6} M or lower, 10^{-7} M or lower, 10^{-8} M or lower, 10^{-9} M or lower, 10^{-10} M or lower, 10^{-11} M or lower, or 10^{-12} M or lower.

An agent is not (substantially) capable of binding to (targeting) a target if it has no significant affinity for said target and does not bind significantly, in particular does not bind detectably, to said target in standard assays. Preferably, the agent does not detectably bind to said target if present in a concentration of up to 2, preferably 10, more preferably 20, in particular 50 or 100 $\mu\text{g/ml}$ or higher. Preferably, an agent has no significant affinity for a target if it binds to said target with a K_D that is at least 10-fold, 100-fold, 10^3 -fold, 10^4 -fold, 10^5 -fold, or 10^6 -fold higher than the K_D for binding to the predetermined target to which the agent is capable of binding. For example, if the K_D for binding of an agent to the target to which the agent is capable of binding is 10^{-7} M, the K_D for binding to a target for which the agent has no significant affinity would be at least 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, or 10^{-1} M.

An agent is specific for a predetermined target if it is capable of binding to said predetermined target while it is not (substantially) capable of binding to other targets, i.e. has no significant affinity for other targets and does not significantly bind to other targets in standard assays. Preferably, an agent is specific for a predetermined target if the affinity for and the binding to such other targets does not significantly exceed the affinity for or binding to proteins which are unrelated to a predetermined target such as bovine serum albumin (BSA), casein or human serum albumin (HSA). Preferably, an agent is specific for a predetermined target if it binds to said target with a K_D that is at least 10-fold, 100-fold, 10^3 -fold, 10^4 -fold, 10^5 -fold, or 10^6 -fold lower than the K_D for binding to a target for which it is not specific. For example, if the K_D for binding of an agent to the target for which it is specific is 10^{-7} M, the K_D for binding to a target for which it is not specific would be at least 10^{-6} M, 10^{-5} M, 10^{-4} M, 10^{-3} M, 10^{-2} M, or 10^{-1} M.

Binding of an agent to a target can be determined experimentally using any suitable method; see, for example, Berzofsky et al., "Antibody-Antigen Interactions" In Fundamental Immunology, Paul, W. E., Ed., Raven Press New York, N Y (1984), Kuby, Janis Immunology, W. H. Freeman and Company New York, N Y (1992), and methods described herein. Affinities may be readily determined using conventional techniques, such as by equilibrium dialysis; by using the BIAcore 2000 instrument, using general procedures outlined by the manufacturer; by radioimmunoassay using radiolabeled target antigen; or by another method known to the skilled artisan. The affinity

data may be analyzed, for example, by the method of Scatchard et al., Ann N.Y. Acad. Sci., 51:660 (1949). The measured affinity of a particular antibody-antigen interaction can vary if measured under different conditions, e.g., salt concentration, pH. Thus, measurements of affinity and other antigen-binding parameters, e.g., K_D , IC_{50} , are preferably made with standardized solutions of antibody and antigen, and a standardized buffer.

The invention may involve introduction, i.e. transfection, of nucleic acids encoding antigen receptors into cells such as T cells *in vitro* or *in vivo*.

For purposes of the present invention, the term "transfection" includes the introduction of a nucleic acid into a cell or the uptake of a nucleic acid by a cell, wherein the cell may be present in a subject, e.g., a patient. Thus, according to the present invention, a cell for transfection of a nucleic acid described herein can be present *in vitro* or *in vivo*, e.g. the cell can form part of an organ, a tissue and/or an organism of a patient. According to the invention, transfection can be transient or stable. For some applications of transfection, it is sufficient if the transfected genetic material is only transiently expressed. Since the nucleic acid introduced in the transfection process is usually not integrated into the nuclear genome, the foreign nucleic acid will be diluted through mitosis or degraded. Cells allowing episomal amplification of nucleic acids greatly reduce the rate of dilution. If it is desired that the transfected nucleic acid actually remains in the genome of the cell and its daughter cells, a stable transfection must occur. RNA can be transfected into cells to transiently express its coded protein.

According to the present invention, any technique useful for introducing, i.e. transferring or transfecting, nucleic acids into cells may be used. Preferably, nucleic acid such as RNA is transfected into cells by standard techniques. Such techniques include electroporation, lipofection and microinjection. In one particularly preferred embodiment of the present invention, RNA is introduced into cells by electroporation. Electroporation or electropermeabilization relates to a significant increase in the electrical conductivity and permeability of the cell plasma membrane caused by an externally applied electrical field. It is usually used in molecular biology as a way of introducing some substance into a cell. According to the invention it is preferred that introduction of nucleic acid encoding a protein or peptide into cells results in expression of said protein or peptide.

A variety of methods may be used to introduce antigen receptor constructs into T cells including

non-viral-based DNA transfection, transposon-based systems and viral-based systems. Non-viral-based DNA transfection has low risk of insertional mutagenesis. Transposon-based systems can integrate transgenes more efficiently than plasmids that do not contain an integrating element. Viral-based systems include the use of γ -retroviruses and lentiviral vectors. γ -Retroviruses are relatively easy to produce, efficiently and permanently transduce T cells, and have preliminarily proven safe from an integration standpoint in primary human T cells. Lentiviral vectors also efficiently and permanently transduce T cells but are more expensive to manufacture. They are also potentially safer than retrovirus based systems.

For transfection of cells *in vivo* a pharmaceutical composition comprising nucleic acid encoding the antigen receptor may be used. A delivery vehicle that targets the nucleic acid to a specific cell such as a T cell may be administered to a patient, resulting in transfection that occurs *in vivo*.

According to the invention it is preferred to administer the nucleic acid encoding an antigen receptor in naked form or in a carrier. The carriers such as lipid carriers contemplated for use in the present invention include any substances or vehicles with which nucleic acid such as RNA can be associated, e.g. by forming complexes with the nucleic acid or forming vesicles in which the nucleic acid is enclosed or encapsulated. This may result in increased stability of the nucleic acid compared to naked nucleic acid. In particular, stability of the nucleic acid in blood may be increased. For example, nanoparticulate RNA formulations with defined particle size, such as lipoplexes from RNA and liposomes, e.g. lipoplexes comprising DOTMA and DOPE or DOTMA and Cholesterol, can be used.

As used herein, the term "nanoparticle" refers to any particle having a diameter making the particle suitable for systemic, in particular parenteral, administration, of, in particular, nucleic acids, typically a diameter of less than 1000 nanometers (nm). In some embodiments, a nanoparticle has a diameter of less than 600 nm. In some embodiments, a nanoparticle has a diameter of less than 400 nm.

As used herein, the term "nanoparticulate formulation" or similar terms refer to any substance that contains at least one nanoparticle. In some embodiments, a nanoparticulate composition is a uniform collection of nanoparticles. In some embodiments, nanoparticulate compositions are dispersions or emulsions. In general, a dispersion or emulsion is formed when at least two immiscible materials are combined.

The term, "lipoplex" or "nucleic acid lipoplex", in particular "RNA lipoplex", refers to a complex of lipids and nucleic acids, in particular RNA. Lipoplexes are formed spontaneously when cationic liposomes, which often also include a neutral "helper" lipid, are mixed with nucleic acids.

Cationic lipids, cationic polymers and other substances with positive charges may form complexes with negatively charged nucleic acids. These cationic molecules can be used to complex nucleic acids, thereby forming e.g. so-called lipoplexes or polyplexes, respectively, and these complexes have been shown to deliver nucleic acids into cells.

Nanoparticulate nucleic acid preparations for use in the present invention can be obtained by various protocols and from various nucleic acid complexing compounds. Lipids, polymers, oligomers, or amphiphiles are typical complexing agents. In one embodiment, the complexing compound comprises at least one agent selected from the group consisting protamine, polyethyleneimine, a poly-L-lysine, a poly-L-arginine or a histone.

According to the invention, protamine is useful as cationic carrier agent. The term "protamine" refers to any of various strongly basic proteins of relatively low molecular weight that are rich in arginine and are found associated especially with DNA in place of somatic histones in the sperm cells of various animals (as fish). In particular, the term "protamine" refers to proteins found in fish sperm that are strongly basic, are soluble in water, are not coagulated by heat, and yield chiefly arginine upon hydrolysis. In purified form, they are used in a long-acting formulation of insulin and to neutralize the anticoagulant effects of heparin.

According to the invention, the term "protamine" as used herein is meant to comprise any protamine amino acid sequence obtained or derived from native or biological sources including fragments thereof and multimeric forms of said amino acid sequence or fragment thereof. Furthermore, the term encompasses (synthesized) polypeptides which are artificial and specifically designed for specific purposes and cannot be isolated from native or biological sources.

The protamine used according to the present invention can be sulfated protamine or hydrochloride protamine. In a preferred embodiment, the protamine source used for the

production of the nanoparticles described herein is protamine 5000 which contains protamine at more than 10 mg/ml (5000 heparin-neutralizing units per ml) in an isotonic salt solution.

Liposomes are microscopic lipidic vesicles often having one or more bilayers of a vesicle-forming lipid, such as a phospholipid, and are capable of encapsulating a drug. Different types of liposomes may be employed in the context of the present invention, including, without being limited thereto, multilamellar vesicles (MLV), small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), sterically stabilized liposomes (SSL), multivesicular vesicles (MV), and large multivesicular vesicles (LMV) as well as other bilayered forms known in the art. The size and lamellarity of the liposome will depend on the manner of preparation and the selection of the type of vesicles to be used will depend on the preferred mode of administration. There are several other forms of supramolecular organization in which lipids may be present in an aqueous medium, comprising lamellar phases, hexagonal and inverse hexagonal phases, cubic phases, micelles, reverse micelles composed of monolayers. These phases may also be obtained in the combination with DNA or RNA, and the interaction with RNA and DNA may substantially affect the phase state. The described phases may be present in the nanoparticulate nucleic acid formulations of the present invention.

For formation of nucleic acid lipoplexes from nucleic acid and liposomes, any suitable method of forming liposomes can be used so long as it provides the envisaged nucleic acid lipoplexes. Liposomes may be formed using standard methods such as the reverse evaporation method (REV), the ethanol injection method, the dehydration-rehydration method (DRV), sonication or other suitable methods.

After liposome formation, the liposomes can be sized to obtain a population of liposomes having a substantially homogeneous size range.

Bilayer-forming lipids have typically two hydrocarbon chains, particularly acyl chains, and a head group, either polar or nonpolar. Bilayer-forming lipids are either composed of naturally-occurring lipids or of synthetic origin, including the phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidic acid, phosphatidylinositol, and sphingomyelin, where the two hydrocarbon chains are typically between about 14-22 carbon atoms in length, and have varying degrees of unsaturation. Other suitable lipids for use in the composition of the present

invention include glycolipids and sterols such as cholesterol and its various analogs which can also be used in the liposomes.

Cationic lipids typically have a lipophilic moiety, such as a sterol, an acyl or diacyl chain, and have an overall net positive charge. The head group of the lipid typically carries the positive charge. The cationic lipid preferably has a positive charge of 1 to 10 valences, more preferably a positive charge of 1 to 3 valences, and more preferably a positive charge of 1 valence. Examples of cationic lipids include, but are not limited to 1,2-di-O-octadecenyl-3-trimethylammonium propane (DOTMA); dimethyldioctadecylammonium (DDAB); 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP); 1,2-dioleoyl-3-dimethylammonium-propane (DODAP); 1,2-diacyloxy-3-dimethylammonium propanes; 1,2-dialkyloxy-3-dimethylammonium propanes; dioctadecyldimethyl ammonium chloride (DODAC), 1,2-dimyristoyloxypropyl-1,3-dimethylhydroxyethyl ammonium (DMRIE), and 2,3-dioleoyloxy-N-[2(spermine carboxamide)ethyl]-N,N-dimethyl-1-propanamium trifluoroacetate (DOSPA). Preferred are DOTMA, DOTAP, DODAC, and DOSPA. Most preferred is DOTMA.

In addition, the nanoparticles described herein preferably further include a neutral lipid in view of structural stability and the like. The neutral lipid can be appropriately selected in view of the delivery efficiency of the nucleic acid-lipid complex. Examples of neutral lipids include, but are not limited to, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), diacylphosphatidyl choline, diacylphosphatidyl ethanol amine, ceramide, sphingoemyelin, cephalin, sterol, and cerebroside. Preferred is DOPE and/or DOPC. Most preferred is DOPE. In the case where a cationic liposome includes both a cationic lipid and a neutral lipid, the molar ratio of the cationic lipid to the neutral lipid can be appropriately determined in view of stability of the liposome and the like.

According to one embodiment, the nanoparticles described herein may comprise phospholipids. The phospholipids may be a glycerophospholipid. Examples of glycerophospholipid include, without being limited thereto, three types of lipids: (i) zwitterionic phospholipids, which include, for example, phosphatidylcholine (PC), egg yolk phosphatidylcholine, soybean-derived PC in natural, partially hydrogenated or fully hydrogenated form, dimyristoyl phosphatidylcholine (DMPC) sphingomyelin (SM); (ii) negatively charged phospholipids: which include, for example, phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidic acid (PA), phosphatidylglycerol (PG) dipalmitoyl PG, dimyristoyl phosphatidylglycerol (DMPG);

synthetic derivatives in which the conjugate renders a zwitterionic phospholipid negatively charged such is the case of methoxy-polyethylene, glycol- distearoyl phosphatidylethanolamine (mPEG-DSPE); and (iii) cationic phospholipids, which include, for example, phosphatidylcholine or sphingomyelin of which the phosphomonoester was O-methylated to form the cationic lipids.

Association of nucleic acid to the lipid carrier can occur, for example, by the nucleic acid filling interstitial spaces of the carrier, such that the carrier physically entraps the nucleic acid, or by covalent, ionic, or hydrogen bonding, or by means of adsorption by non-specific bonds. Whatever the mode of association, the nucleic acid must retain its therapeutic, i.e. encoding, properties.

According to the invention, the nucleic acid encoding an antigen receptor in one embodiment is RNA, preferably mRNA. The RNA is preferably obtained by *in-vitro* transcription.

The term "nucleic acid", as used herein, is intended to include DNA and RNA such as genomic DNA, cDNA, mRNA, recombinantly produced and chemically synthesized molecules. A nucleic acid may be single-stranded or double-stranded. RNA includes *in vitro* transcribed RNA (IVT RNA) or synthetic RNA. According to the invention, a nucleic acid is preferably an isolated nucleic acid.

Nucleic acids may be comprised in a vector. The term "vector" as used herein includes any vectors known to the skilled person including plasmid vectors, cosmid vectors, phage vectors such as lambda phage, viral vectors such as adenoviral or baculoviral vectors, or artificial chromosome vectors such as bacterial artificial chromosomes (BAC), yeast artificial chromosomes (YAC), or P1 artificial chromosomes (PAC). Said vectors include expression as well as cloning vectors. Expression vectors comprise plasmids as well as viral vectors and generally contain a desired coding sequence and appropriate DNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism (e.g., bacteria, yeast, plant, insect, or mammal) or in *in vitro* expression systems. Cloning vectors are generally used to engineer and amplify a certain desired DNA fragment and may lack functional sequences needed for expression of the desired DNA fragments.

In the context of the present invention, the term "RNA" relates to a molecule which comprises

ribonucleotide residues and preferably being entirely or substantially composed of ribonucleotide residues. "Ribonucleotide" relates to a nucleotide with a hydroxyl group at the 2'-position of a β -D-ribofuranosyl group. The term includes double stranded RNA, single stranded RNA, isolated RNA such as partially purified RNA, essentially pure RNA, synthetic RNA, recombinantly produced RNA, as well as modified RNA that differs from naturally occurring RNA by the addition, deletion, substitution and/or alteration of one or more nucleotides. Such alterations can include addition of non-nucleotide material, such as to the end(s) of a RNA or internally, for example at one or more nucleotides of the RNA. Nucleotides in RNA molecules can also comprise non-standard nucleotides, such as non-naturally occurring nucleotides or chemically synthesized nucleotides or deoxynucleotides. These altered RNAs can be referred to as analogs or analogs of naturally-occurring RNA.

According to the present invention, the term "RNA" includes and preferably relates to "mRNA" which means "messenger RNA" and relates to a "transcript" which may be produced using DNA as template and encodes a peptide or protein. mRNA typically comprises a 5' non translated region (5'-UTR), a protein or peptide coding region and a 3' non translated region (3'-UTR). mRNA has a limited halftime in cells and *in vitro*. Preferably, mRNA is produced by *in vitro* transcription using a DNA template. In one embodiment of the invention, the RNA is obtained by *in vitro* transcription or chemical synthesis. The *in vitro* transcription methodology is known to the skilled person. For example, there is a variety of *in vitro* transcription kits commercially available.

In one embodiment of the present invention, RNA is self-replicating RNA, such as single stranded self-replicating RNA. In one embodiment, the self-replicating RNA is single stranded RNA of positive sense. In one embodiment, the self-replicating RNA is viral RNA or RNA derived from viral RNA. In one embodiment, the self-replicating RNA is alphaviral genomic RNA or is derived from alphaviral genomic RNA. In one embodiment, the self-replicating RNA is a viral gene expression vector. In one embodiment, the virus is Semliki forest virus. In one embodiment, the self-replicating RNA contains one or more transgenes at least one of said transgenes encoding the agents described herein. In one embodiment, if the RNA is viral RNA or derived from viral RNA, the transgenes may partially or completely replace viral sequences such as viral sequences encoding structural proteins. In one embodiment, the self-replicating RNA is *in vitro* transcribed RNA.

In order to increase expression and/or stability of the RNA used according to the present invention, it may be modified, preferably without altering the sequence of the expressed peptide or protein.

The term "modification" in the context of RNA as used according to the present invention includes any modification of RNA which is not naturally present in said RNA.

In one embodiment of the invention, the RNA used according to the invention does not have uncapped 5'-triphosphates. Removal of such uncapped 5'-triphosphates can be achieved by treating RNA with a phosphatase.

The RNA according to the invention may have modified naturally occurring or synthetic ribonucleotides in order to increase its stability and/or decrease cytotoxicity. For example, in one embodiment, in the RNA used according to the invention 5-methylcytidine is substituted partially or completely, preferably completely, for cytidine. Alternatively or additionally, in one embodiment, in the RNA used according to the invention pseudouridine is substituted partially or completely, preferably completely, for uridine.

In one embodiment, the term "modification" relates to providing an RNA with a 5'-cap or 5'-cap analog. The term "5'-cap" refers to a cap structure found on the 5'-end of an mRNA molecule and generally consists of a guanosine nucleotide connected to the mRNA via an unusual 5' to 5' triphosphate linkage. In one embodiment, this guanosine is methylated at the 7-position. The term "conventional 5'-cap" refers to a naturally occurring RNA 5'-cap, preferably to the 7-methylguanosine cap (m7G). In the context of the present invention, the term "5'-cap" includes a 5'-cap analog that resembles the RNA cap structure and is modified to possess the ability to stabilize RNA if attached thereto, preferably *in vivo* and/or in a cell.

Providing an RNA with a 5'-cap or 5'-cap analog may be achieved by *in vitro* transcription of a DNA template in the presence of said 5'-cap or 5'-cap analog, wherein said 5'-cap is co-transcriptionally incorporated into the generated RNA strand, or the RNA may be generated, for example, by *in vitro* transcription, and the 5'-cap may be attached to the RNA post-transcriptionally using capping enzymes, for example, capping enzymes of vaccinia virus.

The RNA may comprise further modifications. For example, a further modification of the RNA

used in the present invention may be an extension or truncation of the naturally occurring poly(A) tail or an alteration of the 5'- or 3'-untranslated regions (UTR) such as introduction of a UTR which is not related to the coding region of said RNA, for example, the insertion of one or more, preferably two copies of a 3'-UTR derived from a globin gene, such as alpha2-globin, alpha1-globin, beta-globin, preferably beta-globin, more preferably human beta-globin.

Therefore, in order to increase stability and/or expression of the RNA used according to the present invention, it may be modified so as to be present in conjunction with a poly-A sequence, preferably having a length of 10 to 500, more preferably 30 to 300, even more preferably 65 to 200 and especially 100 to 150 adenosine residues. In an especially preferred embodiment the poly-A sequence has a length of approximately 120 adenosine residues. In addition, incorporation of two or more 3'-non translated regions (UTR) into the 3'-non translated region of an RNA molecule can result in an enhancement in translation efficiency. In one particular embodiment the 3'-UTR is derived from the human β -globin gene.

The term "stability" of RNA relates to the "half-life" of RNA. "Half-life" relates to the period of time which is needed to eliminate half of the activity, amount, or number of molecules. In the context of the present invention, the half-life of an RNA is indicative for the stability of said RNA. The half-life of RNA may influence the "duration of expression" of the RNA. It can be expected that RNA having a long half-life will be expressed for an extended time period.

In the context of the present invention, the term "transcription" relates to a process, wherein the genetic code in a DNA sequence is transcribed into RNA. Subsequently, the RNA may be translated into protein. According to the present invention, the term "transcription" comprises "*in vitro* transcription", wherein the term "*in vitro* transcription" relates to a process wherein RNA, in particular mRNA, is *in vitro* synthesized in a cell-free system, preferably using appropriate cell extracts. Preferably, cloning vectors are applied for the generation of transcripts. These cloning vectors are generally designated as transcription vectors and are according to the present invention encompassed by the term "vector".

The term "translation" according to the invention relates to the process in the ribosomes of a cell by which a strand of messenger RNA directs the assembly of a sequence of amino acids to make a peptide or protein.

Nucleic acids may, according to the invention, be present alone or in combination with other nucleic acids, which may be homologous or heterologous. In preferred embodiments, a nucleic acid is functionally linked to expression control sequences which may be homologous or heterologous with respect to said nucleic acid. The term "homologous" means that the nucleic acids are also functionally linked naturally and the term "heterologous" means that the nucleic acids are not functionally linked naturally.

A nucleic acid and an expression control sequence are "functionally" linked to one another, if they are covalently linked to one another in such a way that expression or transcription of said nucleic acid is under the control or under the influence of said expression control sequence. If the nucleic acid is to be translated into a functional protein, then, with an expression control sequence functionally linked to a coding sequence, induction of said expression control sequence results in transcription of said nucleic acid, without causing a frame shift in the coding sequence or said coding sequence not being capable of being translated into the desired protein or peptide.

The term "expression control sequence" or "expression control element" comprises according to the invention promoters, ribosome binding sites, enhancers and other control elements which regulate transcription of a gene or translation of an mRNA. In particular embodiments of the invention, the expression control sequences can be regulated. The exact structure of expression control sequences may vary as a function of the species or cell type, but generally comprises 5'-untranscribed and 5'- and 3'-untranslated sequences which are involved in initiation of transcription and translation, respectively, such as TATA box, capping sequence, CAAT sequence, and the like. More specifically, 5'-untranscribed expression control sequences comprise a promoter region which includes a promoter sequence for transcriptional control of the functionally linked nucleic acid. Expression control sequences may also comprise enhancer sequences or upstream activator sequences.

The term "expression" is used according to the invention in its most general meaning and comprises the production of RNA and/or peptides or proteins, e.g. by transcription and/or translation. With respect to RNA, the term "expression" or "translation" relates in particular to the production of peptides or proteins. It also comprises partial expression of nucleic acids. Moreover, expression can be transient or stable. According to the invention, the term expression also includes an "aberrant expression" or "abnormal expression".

"Aberrant expression" or "abnormal expression" means according to the invention that expression is altered, preferably increased, compared to a reference, e.g. a state in a subject not having a disease associated with aberrant or abnormal expression of a certain protein, e.g., a tumor antigen. An increase in expression refers to an increase by at least 10%, in particular at least 20%, at least 50% or at least 100%, or more. In one embodiment, expression is only found in a diseased tissue, while expression in a healthy tissue is repressed.

The term "specifically expressed" means that a protein is essentially only expressed in a specific tissue or organ. For example, a tumor antigen specifically expressed in gastric mucosa means that said protein is primarily expressed in gastric mucosa and is not expressed in other tissues or is not expressed to a significant extent in other tissue or organ types. Thus, a protein that is exclusively expressed in cells of the gastric mucosa and to a significantly lesser extent in any other tissue, such as testis, is specifically expressed in cells of the gastric mucosa. In some embodiments, a tumor antigen may also be specifically expressed under normal conditions in more than one tissue type or organ, such as in 2 or 3 tissue types or organs, but preferably in not more than 3 different tissue or organ types. In this case, the tumor antigen is then specifically expressed in these organs. For example, if a tumor antigen is expressed under normal conditions preferably to an approximately equal extent in lung and stomach, said tumor antigen is specifically expressed in lung and stomach.

According to the invention, the term "nucleic acid encoding" means that nucleic acid, if present in the appropriate environment, preferably within a cell, can be expressed to produce a protein or peptide it encodes.

The term "peptide" according to the invention comprises oligo- and polypeptides and refers to substances comprising two or more, preferably 3 or more, preferably 4 or more, preferably 6 or more, preferably 8 or more, preferably 9 or more, preferably 10 or more, preferably 13 or more, preferably 16 more, preferably 21 or more and up to preferably 8, 10, 20, 30, 40 or 50, in particular 100 amino acids joined covalently by peptide bonds. The term "protein" refers to large peptides, preferably to peptides with more than 100 amino acid residues, but in general the terms "peptide", "peptide chain" and "protein" are synonyms and are used interchangeably herein.

As mentioned above, the amino acid sequences of the peptide chains and antigen receptors described herein can be modified so as to obtain variants of said amino acid sequences.

Accordingly, the present invention includes variants of the peptide and protein sequences described herein and includes variants of naturally occurring amino acid sequences resulting in sequences which are functionally equivalent to said sequences, e.g. amino acid sequences exhibiting properties identical or similar to those of said sequences. Important properties are to retain binding of an antigen receptor to its target or transduction of the antigen binding signal to a cell such as a T cell. In one embodiment, a variant molecule or sequence is immunologically equivalent to its parental molecule or sequence.

The term "variant" according to the invention refers, in particular, to mutants, splice variants, conformations, isoforms, allelic variants, species variants and species homologs, in particular those which are naturally present. An allelic variant relates to an alteration in the normal sequence of a gene, the significance of which is often unclear. Complete gene sequencing often identifies numerous allelic variants for a given gene. A species homolog is a nucleic acid or amino acid sequence with a different species of origin from that of a given nucleic acid or amino acid sequence. The term "variant" shall encompass any posttranslationally modified variants and conformation variants.

The term "immunologically equivalent" means that the immunologically equivalent molecule such as the immunologically equivalent amino acid sequence exhibits the same or essentially the same immunological properties and/or exerts the same or essentially the same immunological effects, e.g., with respect to the type of the immunological effect. In the context of the present invention, the term "immunologically equivalent" is preferably used with respect to the immunological effects or properties of antigen receptors used for therapy.

It will be appreciated by those skilled in the art that in particular the sequences of the CDR sequences, hypervariable and variable regions can be modified without losing the ability to bind to a target. For example, CDR regions may be either identical or highly homologous to the regions of parental antibodies. By "highly homologous" it is contemplated that from 1 to 5, preferably from 1 to 4, such as 1 to 3 or 1 or 2 substitutions may be made in the CDRs.

For the purposes of the present invention, "variants" of an amino acid sequence comprise amino acid insertion variants, amino acid addition variants, amino acid deletion variants and/or amino acid substitution variants. Amino acid deletion variants that comprise the deletion at the N-terminal and/or C-terminal end of the protein are also called N-terminal and/or C-terminal

truncation variants.

Amino acid insertion variants comprise insertions of single or two or more amino acids in a particular amino acid sequence. In the case of amino acid sequence variants having an insertion, one or more amino acid residues are inserted into a particular site in an amino acid sequence, although random insertion with appropriate screening of the resulting product is also possible.

Amino acid addition variants comprise amino- and/or carboxy-terminal fusions of one or more amino acids, such as 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids.

Amino acid deletion variants are characterized by the removal of one or more amino acids from the sequence, such as by removal of 1, 2, 3, 5, 10, 20, 30, 50, or more amino acids. The deletions may be in any position of the protein.

Amino acid substitution variants are characterized by at least one residue in the sequence being removed and another residue being inserted in its place. Preference is given to the modifications being in positions in the amino acid sequence which are not conserved between homologous proteins or peptides and/or to replacing amino acids with other ones having similar properties. Preferably, amino acid changes in protein variants are conservative amino acid changes, i.e., substitutions of similarly charged or uncharged amino acids. A conservative amino acid change involves substitution of one of a family of amino acids which are related in their side chains. Naturally occurring amino acids are generally divided into four families: acidic (aspartate, glutamate), basic (lysine, arginine, histidine), non-polar (alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), and uncharged polar (glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine) amino acids. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids.

Preferably the degree of similarity, preferably identity between a given amino acid sequence and an amino acid sequence which is a variant of said given amino acid sequence will be at least about 60%, 65%, 70%, 80%, 81%, 82%, 83%, 84%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99%. The degree of similarity or identity is given preferably for an amino acid region which is at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90% or about 100% of the entire length of the reference amino

acid sequence. For example, if the reference amino acid sequence consists of 200 amino acids, the degree of similarity or identity is given preferably for at least about 20, at least about 40, at least about 60, at least about 80, at least about 100, at least about 120, at least about 140, at least about 160, at least about 180, or about 200 amino acids, preferably continuous amino acids. In preferred embodiments, the degree of similarity or identity is given for the entire length of the reference amino acid sequence. The alignment for determining sequence similarity, preferably sequence identity can be done with art known tools, preferably using the best sequence alignment, for example, using Align, using standard settings, preferably EMBOSS::needle, Matrix: Blosum62, Gap Open 10.0, Gap Extend 0.5.

"Sequence similarity" indicates the percentage of amino acids that either are identical or that represent conservative amino acid substitutions. "Sequence identity" between two amino acid sequences indicates the percentage of amino acids that are identical between the sequences.

The term "percentage identity" is intended to denote a percentage of amino acid residues which are identical between the two sequences to be compared, obtained after the best alignment, this percentage being purely statistical and the differences between the two sequences being distributed randomly and over their entire length. Sequence comparisons between two amino acid sequences are conventionally carried out by comparing these sequences after having aligned them optimally, said comparison being carried out by segment or by "window of comparison" in order to identify and compare local regions of sequence similarity. The optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, *Adv. App. Math.* 2, 482, by means of the local homology algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, *Proc. Natl Acad. Sci. USA* 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

The percentage identity is calculated by determining the number of identical positions between the two sequences being compared, dividing this number by the number of positions compared and multiplying the result obtained by 100 so as to obtain the percentage identity between these two sequences.

Homologous amino acid sequences exhibit according to the invention at least 40%, in particular at least 50%, at least 60%, at least 70%, at least 80%, at least 90% and preferably at least 95%, at least 98 or at least 99% identity of the amino acid residues.

According to the invention, a variant, fragment, part, portion or derivative of an amino acid sequence, peptide or protein preferably has a functional property of the amino acid sequence, peptide or protein, respectively, from which it has been derived, i.e. it is functionally equivalent. In one embodiment, a variant, fragment, part, portion or derivative of an amino acid sequence, peptide or protein is functionally equivalent such as immunologically equivalent to the amino acid sequence, peptide or protein, respectively, from which it has been derived. In one embodiment, the functional property is the property to bind to antigen or transduce the binding signal within a cell.

The term "derived" means according to the invention that a particular entity, in particular a particular sequence, is present in the object from which it is derived, in particular an organism or molecule. In the case of amino acid sequences, especially particular sequence regions, "derived" in particular means that the relevant amino acid sequence is derived from an amino acid sequence in which it is present.

The term "cell" or "host cell" preferably relates to an intact cell, i.e. a cell with an intact membrane that has not released its normal intracellular components such as enzymes, organelles, or genetic material. An intact cell preferably is a viable cell, i.e. a living cell capable of carrying out its normal metabolic functions. Preferably said term relates according to the invention to any cell which can be transfected with an exogenous nucleic acid. Preferably, the cell when transfected with an exogenous nucleic acid and transferred to a recipient can express the nucleic acid in the recipient. The term "cell" includes bacterial cells; other useful cells are yeast cells, fungal cells or mammalian cells. Suitable bacterial cells include cells from gram-negative bacterial strains such as strains of *Escherichia coli*, *Proteus*, and *Pseudomonas*, and gram-positive bacterial strains such as strains of *Bacillus*, *Streptomyces*, *Staphylococcus*, and *Lactococcus*. Suitable fungal cell include cells from species of *Trichoderma*, *Neurospora*, and *Aspergillus*. Suitable yeast cells include cells from species of *Saccharomyces* (for example *Saccharomyces cerevisiae*), *Schizosaccharomyces* (for example *Schizosaccharomyces pombe*), *Pichia* (for example *Pichia pastoris* and *Pichia methanolicus*), and *Hansenula*. Suitable mammalian cells include for example CHO cells, BHK cells, HeLa cells, COS cells, 293 HEK

and the like. However, amphibian cells, insect cells, plant cells, and any other cells used in the art for the expression of heterologous proteins can be used as well. Mammalian cells are particularly preferred for adoptive transfer, such as cells from humans, mice, hamsters, pigs, goats, and primates. The cells may be derived from a large number of tissue types and include primary cells and cell lines such as cells of the immune system, in particular antigen-presenting cells such as dendritic cells and T cells, stem cells such as hematopoietic stem cells and mesenchymal stem cells and other cell types. Particularly preferred cells for use according to the invention are immunoreactive or immune effector cells, in particular T cells.

A cell which comprises a nucleic acid molecule preferably express the peptide or protein encoded by the nucleic acid.

The term "clonal expansion" or "expansion" refers to a process wherein a specific entity is multiplied. In the context of the present invention, the term is preferably used in the context of an immunological response in which lymphocytes are stimulated by an antigen, proliferate, and the specific lymphocyte recognizing said antigen is amplified. Preferably, clonal expansion leads to differentiation of the lymphocytes. The term "priming" refers to a process wherein a T cell has its first contact with its specific antigen and causes differentiation into effector T cells.

The molecules such as nucleic acids, peptide chains or antigen receptors, or cells described herein may be recombinant and/or isolated.

The term "isolated" as used herein, is intended to refer to an entity which is substantially free of other molecules such as other cellular material. The term "isolated" preferably means that the isolated entity has been separated from its natural environment. An isolated entity may be in an essentially purified state. The term "essentially purified" preferably means that the entity is essentially free of other substances with which it is associated in nature or *in vivo*.

The term "recombinant" in the context of the present invention means "made through genetic engineering". Preferably, a "recombinant object" such as a recombinant cell in the context of the present invention is not occurring naturally.

The term "naturally occurring" as used herein refers to the fact that an object can be found in nature. For example, a peptide or nucleic acid that is present in an organism (including viruses)

and can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally occurring.

The term "autologous" is used to describe anything that is derived from the same subject. For example, "autologous transplant" refers to a transplant of tissue or organs derived from the same subject. Such procedures are advantageous because they overcome the immunological barrier which otherwise results in rejection.

The term "allogeneic" is used to describe anything that is derived from different individuals of the same species. Two or more individuals are said to be allogeneic to one another when the genes at one or more loci are not identical.

The term "syngeneic" is used to describe anything that is derived from individuals or tissues having identical genotypes, i.e., identical twins or animals of the same inbred strain, or their tissues.

The term "heterologous" is used to describe something consisting of multiple different elements. As an example, the transfer of one individual's bone marrow into a different individual constitutes a heterologous transplant. A heterologous gene is a gene derived from a source other than the subject.

"Reduce" or "inhibit" as used herein means the ability to cause an overall decrease, preferably of 5% or greater, 10% or greater, 20% or greater, more preferably of 50% or greater, and most preferably of 75% or greater, in the level. The term "inhibit" or similar phrases includes a complete or essentially complete inhibition, i.e. a reduction to zero or essentially to zero.

Terms such as "increase" or "enhance" preferably relate to an increase or enhancement by about at least 10%, preferably at least 20%, preferably at least 30%, more preferably at least 40%, more preferably at least 50%, even more preferably at least 80%, and most preferably at least 100%.

Since an antigen receptor of the present invention can be engineered to target virtually any antigen, including disease-specific antigens, an antigen receptor of the present invention has a broad therapeutic use. Accordingly, the present invention is directed to the use of an antigen

receptor of the invention, its peptide chains, nucleic acids encoding same, and other related molecules in therapeutic and prophylactic methods. One such use is in the production of antigen-specific immune cells, which can be administered to a patient for preventing or treating a disease, which disease is characterized by expression of one or more antigens that can be bound by an antigen receptor of the invention expressed in the immune cells. Preferably, the disease is cancer. Further, an antigen receptor of the invention and related molecules also can be used for the selective eradication of cells expressing a predetermined antigen, as well as for immunization or vaccination against a disease wherein a predetermined antigen is expressed, which antigen can be bound by at least one antigen binding site of an antigen receptor of the invention.

In one embodiment, a method of treating or preventing a disease comprises administering to a patient an effective amount of a nucleic acid encoding an antigen receptor of the invention, in which at least one antigen binding site of the antigen receptor is able to bind an antigen that is associated with the disease (e.g., a viral or tumor antigen) to be treated or prevented. In another embodiment, a method of treating or preventing a disease comprises administering to a patient an effective amount of a recombinant immune effector cell or an expanded population of said immune effector cells, which immune effector cell or population of cells recombinantly express an antigen receptor of the invention, in which at least one antigen binding site of the antigen receptor is able to bind an antigen that is associated with the disease to be treated or prevented. In preferred embodiments, the disease is cancer and the antigen is a tumor associated antigen.

In another embodiment, the present invention provides for a method of immunizing or vaccinating against a disease associated with a specific antigen or against a disease-causing organism expressing a specific antigen, which method comprises administering to a patient an effective amount of a nucleic acid encoding an antigen receptor of the invention, in which at least one antigen binding site of the antigen receptor is able to bind the specific antigen. In another embodiment, the present invention provides for a method of immunizing or vaccinating against a disease associated with a specific antigen or against a disease-causing organism expressing a specific antigen, which method comprises administering to a patient an effective amount of a recombinant immune effector cell or an expanded population of said immune effector cells, which immune effector cell or population of cells recombinantly express an antigen receptor of the invention, in which at least one antigen binding site of the antigen receptor is able to bind to the specific antigen.

In certain embodiments, the population of immune effector cells can be a clonally expanded population. The recombinant immune effector cells or populations thereof provide for therapeutic or prophylactic immune effector function in an antigen-specific manner. Preferably, an antigen receptor of the invention is expressed on the cell surface of the immune effector cell.

The cells used in connection with the therapeutic and prophylactic methods of the present invention are preferably immune effector cells and the immune effector cells are preferably T cells. In particular, the cells used herein are cytotoxic lymphocytes, preferably selected from cytotoxic T cells, natural killer (NK) cells, and lymphokine-activated killer (LAK) cells. Upon activation/stimulation, each of these cytotoxic lymphocytes triggers the destruction of target cells. For example, cytotoxic T cells trigger the destruction of target cells by either or both of the following means. First, upon activation, the T cells release cytotoxins such as perforin, granzymes, and granulysin. Perforin and granulysin create pores in the target cell, and granzymes enter the cell and trigger a caspase cascade in the cytoplasm that induces apoptosis (programmed cell death) of the cell. Second, apoptosis can be induced via Fas-Fas ligand interaction between the T cells and target tumor cells. The T cells and other cytotoxic lymphocytes will preferably be autologous cells, although heterologous cells or allogenic cells can be used.

Accordingly, the agents, compositions and methods described herein can be used to treat a subject with a disease, e.g., a disease characterized by the presence of diseased cells expressing an antigen. Particularly preferred diseases are cancer diseases.

The agents, compositions and methods described herein may also be used for immunization or vaccination to prevent a disease described herein.

The term "disease" refers to an abnormal condition that affects the body of an individual. A disease is often construed as a medical condition associated with specific symptoms and signs. A disease may be caused by factors originally from an external source, such as infectious disease, or it may be caused by internal dysfunctions, such as autoimmune diseases. In humans, "disease" is often used more broadly to refer to any condition that causes pain, dysfunction, distress, social problems, or death to the individual afflicted, or similar problems for those in contact with the individual. In this broader sense, it sometimes includes injuries, disabilities, disorders, syndromes, infections, isolated symptoms, deviant behaviors, and atypical variations of structure

and function, while in other contexts and for other purposes these may be considered distinguishable categories. Diseases usually affect individuals not only physically, but also emotionally, as contracting and living with many diseases can alter one's perspective on life, and one's personality. According to the invention, the term "disease" includes infectious diseases and cancer diseases, in particular those forms of cancer described herein. Any reference herein to cancer or particular forms of cancer also includes cancer metastasis thereof.

A disease to be treated according to the invention is preferably a disease involving an antigen. "Disease involving an antigen", "disease associated with expression or elevated expression of an antigen" or similar expressions means according to the invention that the antigen is expressed in cells of a diseased tissue or organ. Expression in cells of a diseased tissue or organ may be increased compared to the state in a healthy tissue or organ. In one embodiment, expression is only found in a diseased tissue, while expression in a healthy tissue is not found, e.g. expression is repressed. According to the invention, diseases involving an antigen include infectious diseases and cancer diseases, wherein the disease-associated antigen is preferably an antigen of the infectious agent and a tumor antigen, respectively. Preferably a disease involving an antigen preferably is a disease involving cells expressing an antigen, preferably on the cell surface.

The term "healthy" or "normal" refer to non-pathological conditions, and preferably means non-infected or non-cancerous.

The terms "cancer disease" or "cancer" refer to or describe the physiological condition in an individual that is typically characterized by unregulated cell growth. Examples of cancers include, but are not limited to, carcinoma, lymphoma, blastoma, sarcoma, and leukemia. More particularly, examples of such cancers include bone cancer, blood cancer, lung cancer, liver cancer, pancreatic cancer, skin cancer, cancer of the head or neck, cutaneous or intraocular melanoma, uterine cancer, ovarian cancer, rectal cancer, cancer of the anal region, stomach cancer, colon cancer, breast cancer, prostate cancer, uterine cancer, carcinoma of the sexual and reproductive organs, Hodgkin's Disease, cancer of the esophagus, cancer of the small intestine, cancer of the endocrine system, cancer of the thyroid gland, cancer of the parathyroid gland, cancer of the adrenal gland, sarcoma of soft tissue, cancer of the bladder, cancer of the kidney, renal cell carcinoma, carcinoma of the renal pelvis, neoplasms of the central nervous system (CNS), neuroectodermal cancer, spinal axis tumors, glioma, meningioma, and pituitary adenoma. The term "cancer" according to the invention also comprises cancer metastases. Preferably, a

"cancer disease" is characterized by cells expressing a tumor antigen and a cancer cell expresses a tumor antigen.

In one embodiment, a cancer disease is a malignant disease which is characterized by the properties of anaplasia, invasiveness, and metastasis. A malignant tumor may be contrasted with a non-cancerous benign tumor in that a malignancy is not self-limited in its growth, is capable of invading into adjacent tissues, and may be capable of spreading to distant tissues (metastasizing), while a benign tumor has none of those properties.

According to the invention, the term "tumor" or "tumor disease" refers to a swelling or lesion formed by an abnormal growth of cells (called neoplastic cells or tumor cells). By "tumor cell" is meant an abnormal cell that grows by a rapid, uncontrolled cellular proliferation and continues to grow after the stimuli that initiated the new growth cease. Tumors show partial or complete lack of structural organization and functional coordination with the normal tissue, and usually form a distinct mass of tissue, which may be either benign, pre-malignant or malignant.

According to the invention, a "carcinoma" is a malignant tumor derived from epithelial cells. This group represents the most common cancers, including the common forms of breast, prostate, lung and colon cancer.

"Adenocarcinoma" is a cancer that originates in glandular tissue. This tissue is also part of a larger tissue category known as epithelial tissue. Epithelial tissue includes skin, glands and a variety of other tissue that lines the cavities and organs of the body. Epithelium is derived embryologically from ectoderm, endoderm and mesoderm. To be classified as adenocarcinoma, the cells do not necessarily need to be part of a gland, as long as they have secretory properties. This form of carcinoma can occur in some higher mammals, including humans. Well differentiated adenocarcinomas tend to resemble the glandular tissue that they are derived from, while poorly differentiated may not. By staining the cells from a biopsy, a pathologist will determine whether the tumor is an adenocarcinoma or some other type of cancer. Adenocarcinomas can arise in many tissues of the body due to the ubiquitous nature of glands within the body. While each gland may not be secreting the same substance, as long as there is an exocrine function to the cell, it is considered glandular and its malignant form is therefore named adenocarcinoma. Malignant adenocarcinomas invade other tissues and often metastasize given enough time to do so. Ovarian adenocarcinoma is the most common type of ovarian

carcinoma. It includes the serous and mucinous adenocarcinomas, the clear cell adenocarcinoma and the endometrioid adenocarcinoma.

Lymphoma and leukemia are malignancies derived from hematopoietic (blood-forming) cells.

Blastic tumor or blastoma is a tumor (usually malignant) which resembles an immature or embryonic tissue. Many of these tumors are most common in children.

By "metastasis" is meant the spread of cancer cells from its original site to another part of the body. The formation of metastasis is a very complex process and depends on detachment of malignant cells from the primary tumor, invasion of the extracellular matrix, penetration of the endothelial basement membranes to enter the body cavity and vessels, and then, after being transported by the blood, infiltration of target organs. Finally, the growth of a new tumor at the target site depends on angiogenesis. Tumor metastasis often occurs even after the removal of the primary tumor because tumor cells or components may remain and develop metastatic potential. In one embodiment, the term "metastasis" according to the invention relates to "distant metastasis" which relates to a metastasis which is remote from the primary tumor and the regional lymph node system. In one embodiment, the term "metastasis" according to the invention relates to lymph node metastasis.

A relapse or recurrence occurs when a person is affected again by a condition that affected them in the past. For example, if a patient has suffered from a tumor disease, has received a successful treatment of said disease and again develops said disease said newly developed disease may be considered as relapse or recurrence. However, according to the invention, a relapse or recurrence of a tumor disease may but does not necessarily occur at the site of the original tumor disease. Thus, for example, if a patient has suffered from ovarian tumor and has received a successful treatment a relapse or recurrence may be the occurrence of an ovarian tumor or the occurrence of a tumor at a site different to ovary. A relapse or recurrence of a tumor also includes situations wherein a tumor occurs at a site different to the site of the original tumor as well as at the site of the original tumor. Preferably, the original tumor for which the patient has received a treatment is a primary tumor and the tumor at a site different to the site of the original tumor is a secondary or metastatic tumor.

Infectious diseases that can be treated or prevented by the present invention are caused by

infectious agents including, but not limited to, viruses, bacteria, fungi, protozoa, helminths, and parasites.

Infectious viruses of both human and non-human vertebrates, include retroviruses, RNA viruses and DNA viruses. Examples of virus that have been found in humans include but are not limited to: *Retroviridae* (e.g., human immunodeficiency viruses, such as HIV-1 (also referred to as HTLV-III, LAV or HTLV-III/LAV, or HIV-III; and other isolates, such as HIV-LP; *Picornaviridae* (e.g., polio viruses, hepatitis A virus; enteroviruses, human Coxsackie viruses, rhinoviruses, echoviruses); *Calciviridae* (e.g., strains that cause gastroenteritis); *Togaviridae* (e.g., equine encephalitis viruses, rubella viruses); *Flaviridae* (e.g., dengue viruses, encephalitis viruses, yellow fever viruses); *Coronaviridae* (e.g., coronaviruses); *Rhabdoviridae* (e.g., vesicular stomatitis viruses, rabies viruses); *Filoviridae* (e.g., ebola viruses); *Paramyxoviridae* (e.g., parainfluenza viruses, mumps virus, measles virus, respiratory syncytial virus); *Orthomyxoviridae* (e.g., influenza viruses); *Bungaviridae* (e.g., Hanta viruses, bunga viruses, phleboviruses and Nairo viruses); *Arena viridae* (hemorrhagic fever viruses); *Reoviridae* (e.g., reoviruses, orbiviruses and rotaviruses); *Birnaviridae*; *Hepadnaviridae* (Hepatitis B virus); *Parvovirida* (parvoviruses); *Papovaviridae* (papilloma viruses, polyoma viruses); *Adenoviridae* (most adenoviruses); *Herpesviridae* (herpes simplex virus (HSV) 1 and 2, varicella zoster virus, cytomegalovirus (CMV), herpes virus; *Poxviridae* (variola viruses, vaccinia viruses, pox viruses); and *Iridoviridae* (e.g., African swine fever virus); and unclassified viruses (e.g., the etiological agents of Spongiform encephalopathies, the agent of delta hepatitis (thought to be a defective satellite of hepatitis B virus), the agents of non-A, non-B hepatitis (class 1=internally transmitted; class 2=parenterally transmitted (*i.e.*, Hepatitis C); Norwalk and related viruses, and astroviruses).

Retroviruses that are contemplated include both simple retroviruses and complex retroviruses. The complex retroviruses include the subgroups of lentiviruses, T cell leukemia viruses and the foamy viruses. Lentiviruses include HIV-1, but also include HIV-2, SIV, Visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV). The T cell leukemia viruses include HTLV-1, HTLV-II, simian T cell leukemia virus (STLV), and bovine leukemia virus (BLV). The foamy viruses include human foamy virus (HFV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Bacterial infections or diseases that can be treated or prevented by the present invention are

caused by bacteria including, but not limited to, bacteria that have an intracellular stage in its life cycle, such as mycobacteria (e.g., *Mycobacteria tuberculosis*, *M. bovis*, *M. avium*, *M. leprae*, or *M. africanum*), rickettsia, mycoplasma, chlamydia, and legionella. Other examples of bacterial infections contemplated include but are not limited to infections caused by Gram positive bacillus (e.g., *Listeria*, *Bacillus* such as *Bacillus anthracis*, *Erysipelothrix* species), Gram negative bacillus (e.g., *Bartonella*, *Brucella*, *Campylobacter*, *Enterobacter*, *Escherichia*, *Francisella*, *Hemophilus*, *Klebsiella*, *Morganella*, *Proteus*, *Providencia*, *Pseudomonas*, *Salmonella*, *Serratia*, *Shigella*, *Vibrio*, and *Yersinia* species), spirochete bacteria (e.g., *Borrelia* species including *Borrelia burgdorferi* that causes Lyme disease), anaerobic bacteria (e.g., *Actinomyces* and *Clostridium* species), Gram positive and negative coccal bacteria, *Enterococcus* species, *Streptococcus* species, *Pneumococcus* species, *Staphylococcus* species, *Neisseria* species. Specific examples of infectious bacteria include but are not limited to: *Helicobacter pylori*, *Borelia burgdorferi*, *Legionella pneumophila*, *Mycobacteria tuberculosis*, *M. avium*, *M. intracellulare*, *M. kansasii*, *M. gordonae*, *Staphylococcus aureus*, *Neisseria gonorrhoeae*, *Neisseria meningitidis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (Group A Streptococcus), *Streptococcus agalactiae* (Group B Streptococcus), *Streptococcus viridans*, *Streptococcus aecalis*, *Streptococcus bovis*, *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Bacillus anthracis*, *Corynebacterium diphtheriae*, *Erysipelothrix rhusiopathiae*, *Clostridium perfringens*, *Clostridium tetani*, *Enterobacter aerogenes*, *Klebsiella pneumoniae*, *Pasturella multocida*, *Fusobacterium nucleatum*, *Streptobacillus moniliformis*, *Treponema pallidum*, *Treponema pertenue*, *Leptospira*, *Rickettsia*, and *Actinomyces israelii*.

Fungal diseases that can be treated or prevented by the present invention include but are not limited to aspergilliosis, cryptococcosis, sporotrichosis, coccidioidomycosis, paracoccidioidomycosis, histoplasmosis, blastomycosis, zygomycosis, and candidiasis.

Parasitic diseases that can be treated or prevented by the present invention include, but are not limited to, amebiasis, malaria, leishmania, coccidia, giardiasis, cryptosporidiosis, toxoplasmosis, and trypanosomiasis. Also encompassed are infections by various worms, such as but not limited to ascariasis, ancylostomiasis, trichuriasis, strongyloidiasis, toxocariasis, trichinosis, onchocerciasis, filaria, and dirofilariasis. Also encompassed are infections by various flukes, such as but not limited to schistosomiasis, paragonimiasis, and clonorchiasis.

The term "treatment" or "therapeutic treatment" relates to any treatment which improves the health status and/or prolongs (increases) the lifespan of an individual. Said treatment may eliminate the disease in an individual, arrest or slow the development of a disease in an individual, inhibit or slow the development of a disease in an individual, decrease the frequency or severity of symptoms in an individual, and/or decrease the recurrence in an individual who currently has or who previously has had a disease.

The terms "prophylactic treatment" or "preventive treatment" relate to any treatment that is intended to prevent a disease from occurring in an individual. The terms "prophylactic treatment" or "preventive treatment" are used herein interchangeably.

The terms "individual" and "subject" are used herein interchangeably. They refer to human beings, non-human primates or other mammals (e.g. mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate) that can be afflicted with or are susceptible to a disease or disorder (e.g., cancer) but may or may not have the disease or disorder. In many embodiments, the individual is a human being. Unless otherwise stated, the terms "individual" and "subject" do not denote a particular age, and thus encompass adults, elderlies, children, and newborns. In preferred embodiments of the present invention, the "individual" or "subject" is a "patient". The term "patient" means according to the invention a subject for treatment, in particular a diseased subject.

By "being at risk" is meant a subject, i.e. a patient, that is identified as having a higher than normal chance of developing a disease, in particular cancer, compared to the general population. In addition, a subject who has had, or who currently has, a disease, in particular cancer is a subject who has an increased risk for developing a disease, as such a subject may continue to develop a disease. Subjects who currently have, or who have had, a cancer also have an increased risk for cancer metastases.

The term "immunotherapy" relates to a treatment involving a specific immune reaction or response.

In the context of the present invention, terms such as "protect", "prevent", "prophylactic", "preventive", or "protective" relate to the prevention or treatment or both of the occurrence and/or the propagation of a disease in a subject and, in particular, to minimizing the chance that a

subject will develop a disease or to delaying the development of a disease. For example, a person at risk for a tumor, as described above, would be a candidate for therapy to prevent a tumor.

A prophylactic administration of an immunotherapy, for example, a prophylactic administration of an agent or composition of the invention, preferably protects the recipient from the development of a disease. A therapeutic administration of an immunotherapy, for example, a therapeutic administration of an agent or composition of the invention, may lead to the inhibition of the progress/growth of the disease. This comprises the deceleration of the progress/growth of the disease, in particular a disruption of the progression of the disease, which preferably leads to elimination of the disease.

Immunotherapy may be performed using any of a variety of techniques, in which agents provided herein preferably function to remove antigen-expressing cells from a patient. Such removal may take place as a result of enhancing or inducing an immune response in a patient specific for antigen or a cell expressing antigen.

The term "immunization" or "vaccination" describes the process of treating a subject with the purpose of inducing an immune response for therapeutic or prophylactic reasons.

The term "*in vivo*" relates to the situation in a subject.

The antigen receptors, peptide chains, nucleic acids, recombinant cells, immune effector cells, preferably T cells, of the invention, as well as other compounds and agents described herein may be administered in the form of any suitable pharmaceutical composition.

The pharmaceutical compositions of the invention are preferably sterile and contain an effective amount of the agents described herein and optionally of further agents as discussed herein to generate the desired reaction or the desired effect.

Pharmaceutical compositions are usually provided in a uniform dosage form and may be prepared in a manner known per se. A pharmaceutical composition may e.g. be in the form of a solution or suspension.

A pharmaceutical composition may comprise salts, buffer substances, preservatives, carriers, diluents and/or excipients all of which are preferably pharmaceutically acceptable. The term "pharmaceutically acceptable" refers to the non-toxicity of a material which does not interact with the action of the active component of the pharmaceutical composition.

Salts which are not pharmaceutically acceptable may be used for preparing pharmaceutically acceptable salts and are included in the invention. Pharmaceutically acceptable salts of this kind comprise in a non limiting way those prepared from the following acids: hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic acids, and the like. Pharmaceutically acceptable salts may also be prepared as alkali metal salts or alkaline earth metal salts, such as sodium salts, potassium salts or calcium salts.

Suitable buffer substances for use in a pharmaceutical composition include acetic acid in a salt, citric acid in a salt, boric acid in a salt and phosphoric acid in a salt.

Suitable preservatives for use in a pharmaceutical composition include benzalkonium chloride, chlorobutanol, paraben and thimerosal.

An injectible formulation may comprise a pharmaceutically acceptable excipient such as Ringer Lactate.

The term "carrier" refers to an organic or inorganic component, of a natural or synthetic nature, in which the active component is combined in order to facilitate, enhance or enable application. According to the invention, the term "carrier" also includes one or more compatible solid or liquid fillers, diluents or encapsulating substances, which are suitable for administration to a patient.

Possible carrier substances for parenteral administration are e.g. sterile water, Ringer, Ringer lactate, sterile sodium chloride solution, polyalkylene glycols, hydrogenated naphthalenes and, in particular, biocompatible lactide polymers, lactide/glycolide copolymers or polyoxyethylene/polyoxypropylene copolymers.

The term "excipient" when used herein is intended to indicate all substances which may be present in a pharmaceutical composition and which are not active ingredients such as, e.g.,

carriers, binders, lubricants, thickeners, surface active agents, preservatives, emulsifiers, buffers, flavoring agents, or colorants.

The agents and compositions described herein may be administered via any conventional route, such as by parenteral administration including by injection or infusion. Administration is preferably parenterally, e.g. intravenously, intraarterially, subcutaneously, intradermally or intramuscularly.

Compositions suitable for parenteral administration usually comprise a sterile aqueous or nonaqueous preparation of the active compound, which is preferably isotonic to the blood of the recipient. Examples of compatible carriers and solvents are Ringer solution and isotonic sodium chloride solution. In addition, usually sterile, fixed oils are used as solution or suspension medium.

The agents and compositions described herein are administered in effective amounts. An "effective amount" refers to the amount which achieves a desired reaction or a desired effect alone or together with further doses. In the case of treatment of a particular disease or of a particular condition, the desired reaction preferably relates to inhibition of the course of the disease. This comprises slowing down the progress of the disease and, in particular, interrupting or reversing the progress of the disease. The desired reaction in a treatment of a disease or of a condition may also be delay of the onset or a prevention of the onset of said disease or said condition.

An effective amount of an agent or composition described herein will depend on the condition to be treated, the severeness of the disease, the individual parameters of the patient, including age, physiological condition, size and weight, the duration of treatment, the type of an accompanying therapy (if present), the specific route of administration and similar factors. Accordingly, the doses administered of the agents described herein may depend on various of such parameters. In the case that a reaction in a patient is insufficient with an initial dose, higher doses (or effectively higher doses achieved by a different, more localized route of administration) may be used.

The agents and compositions described herein can be administered to patients, e.g., *in vivo*, to treat or prevent a variety of disorders such as those described herein. Preferred patients include human patients having disorders that can be corrected or ameliorated by administering the agents

and compositions described herein. This includes disorders involving cells characterized by expression of an antigen.

For example, in one embodiment, agents described herein can be used to treat a patient with a cancer disease, e.g., a cancer disease such as described herein characterized by the presence of cancer cells expressing an antigen.

The pharmaceutical compositions and methods of treatment described according to the invention may also be used for immunization or vaccination to prevent a disease described herein.

The pharmaceutical composition of the invention may be administered together with supplementing immunity-enhancing substances such as one or more adjuvants and may comprise one or more immunity-enhancing substances to further increase its effectiveness, preferably to achieve a synergistic effect of immunostimulation. The term "adjuvant" relates to compounds which prolongs or enhances or accelerates an immune response. Various mechanisms are possible in this respect, depending on the various types of adjuvants. For example, compounds which allow the maturation of the DC, e.g. lipopolysaccharides or CD40 ligand, form a first class of suitable adjuvants. Generally, any agent which influences the immune system of the type of a "danger signal" (LPS, GP96, dsRNA etc.) or cytokines, such as GM-CSF, can be used as an adjuvant which enables an immune response to be intensified and/or influenced in a controlled manner. CpG oligodeoxynucleotides can optionally also be used in this context, although their side effects which occur under certain circumstances, as explained above, are to be considered. Particularly preferred adjuvants are cytokines, such as monokines, lymphokines, interleukins or chemokines, e.g. IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IFN α , IFN γ , GM-CSF, LT- α , or growth factors, e.g. hGH. Further known adjuvants are aluminium hydroxide, Freund's adjuvant or oil such as Montanide®, most preferred Montanide® ISA51. Lipopeptides, such as Pam3Cys, are also suitable for use as adjuvants in the pharmaceutical composition of the present invention.

The pharmaceutical composition can be administered locally or systemically, preferably systemically.

The term "systemic administration" refers to the administration of an agent such that the agent becomes widely distributed in the body of an individual in significant amounts and develops a

desired effect. For example, the agent may develop its desired effect in the blood and/or reaches its desired site of action via the vascular system. Typical systemic routes of administration include administration by introducing the agent directly into the vascular system or oral, pulmonary, or intramuscular administration wherein the agent is adsorbed, enters the vascular system, and is carried to one or more desired site(s) of action via the blood.

According to the present invention, it is preferred that the systemic administration is by parenteral administration. The term "parenteral administration" refers to administration of an agent such that the agent does not pass the intestine. The term "parenteral administration" includes intravenous administration, subcutaneous administration, intradermal administration or intraarterial administration but is not limited thereto.

Administration may also be carried out, for example, orally, intraperitoneally or intramuscularly.

The agents and compositions provided herein may be used alone or in combination with conventional therapeutic regimens such as surgery, irradiation, chemotherapy and/or bone marrow transplantation (autologous, syngeneic, allogeneic or unrelated).

The present invention is described in detail by the figures and examples below, which are used only for illustration purposes and are not meant to be limiting. Owing to the description and the examples, further embodiments which are likewise included in the invention are accessible to the skilled worker.

FIGURES

Figure 1 contains a representation of the TCR-CD3 complex. The TCR and the CD3-subunits are composed of ectodomains, a stalk region, a transmembrane domain and cytosolic domains which bear the ITAMs. Expression of the CD4 or CD8 coreceptor determines the commitment to CD4⁺ or CD8⁺ T-cell subsets. The intracytoplasmic CD3 immunoreceptor tyrosine-based activation motifs (ITAMs) are indicated as cylinders (adapted from "The T cell receptor facts book", MP Lefranc, G Lefranc, 2001).

Figure 2 shows the design of successive generations of CARs. Schematic representation of the different generations of CARs (1G, first generation, 2G, second generation, 3G, third

generation). The first generation contains extracellular scFv's and the cytoplasmic CD3 ζ chain/ZAP70 mediating immediate effector function such as IFN γ -secretion or cytotoxicity, the second generation additionally CD28/PI3K promoting proliferation and the third generation furthermore 4-1BB or OX40/TRAF sustaining cell survival (Casucci, M. et al. (2011) 2: 378-382).

Figure 3 contains a schematic representation of the different receptor formats for the redirection of T cells against an antigen. C α / β are the constant domains of a TCR that also operate as a signal transmission domain in relaying the signal of a bound antigen through the cell membrane to the signaling domains of the recruited CD3-complex in the cytoplasm; V H and V L are the variable regions of the heavy and light chains of an immunoglobulin, respectively, and which are representative of the domains on the two peptide chains that form an antigen binding site.

Left: a second generation CAR consisting of an antigen-specific scFv fragment, a IgG1-derived spacer domain, a CD28 costimulatory and a CD3 ζ signaling domain (Classical single chain CAR); middle: a novel CAR format based on the linkage of the scFv with the constant domain of the murine TCR β chain and coexpression of the constant domain of the murine TCR α chain (monovalent non-combinatory antigen receptor); right: a murine TCR composed of TCR α / β chains (mu, murine). The heterodimer CD3 $\delta\epsilon$ and the homodimer CD3 $\zeta\zeta$ is recruited by the C α -domain while CD3 $\gamma\epsilon$ is recruited by the C β -domain.

Figures 4A and B show the structures of certain of the antigen receptors described herein.

Nomenclature of the domains is as described in the legend to figure 3. A tandem antigen receptor (tandem AR) comprises a chain comprising 4 variable antibody fragments which form 2 antigen binding sites. An intra/inter-combinatory AR is able of intra- and interchain-binding to antigens. An inter-combinatory AR allows binding of antigens exclusively via interchain-binding. The monovalent non-combinatory AR represents the monovalent prototype of the novel AR-design based on the recruitment of endogenous CD3, the bivalent non-combinatory AR is a CAR, which design has already been suggested by Gross et al., 1992 FASEB J.,(6) 3370-3378 and confines binding to each antigen to a single chain. The monovalent combinatory AR serves as a reference AR or negative control to prove the benefit in function by providing higher valencies by means of scFv-fragments in CAR-design.

Figure 5 is a histogram showing the relative expression levels of antigen receptors in T cells. Expression on CAR RNA electroporated T-cells has been validated in flow cytometry analysis by taking advantage of an idiotype antibody directed against the paratope of the Claudin 6-specific antibody IMAB206. The flow cytometry antibody was directly labeled with Dylight-640, expression levels are given in mean fluorescence intensities (MFI).

Figures 6A to 6C are histograms showing the relative induced levels of IFN- γ production, which is an indicator of immune cell activation. Figure 6A: Detection of IFN- γ production in ELISA for CD8⁺ T cells electroporated with different antigen receptor expression constructs and controls, and co-cultured with Cl6 negative and positive immature dendritic cells (iDC). The bivalent antigen receptors capable of inter-chain antigen binding showed good IFN- γ production (inter-combinatory AR 2GS, 3GS, 4GS). Variation of the linker length between the variable domains did not have a significant impact on receptor function, but the 3GS linker appeared to be somewhat better compared to the 2- and 4GS-linker. Truncation of the N-terminal variable domains of the bivalent antigen receptor to obtain a monovalent antigen receptor (monovalent combinatory AR) drastically reduced receptor function, demonstrating the importance of bivalent antigen-binding for superior receptor function. Murine constant domains in the antigen receptor structure lead to slightly improved IFN- γ production (inter-comb. AR Mu 3GS). Figure 6B: A replicate experiment of that disclosed in Figure 6A showing similar results but with T cells obtained from a different donor to prove donor-independency of the results. Figure 6C: Essentially a repeat of the experiments disclosed in Figures 6A and 6B. The tandem antigen receptor, and the intra/inter-combinatory antigen receptor (enabling antigen recognition via both intra- and inter-chain VH/VL-domain combination), each lead to a higher induction of IFN- γ expression compared to the monovalent antigen receptor (monovalent non-combinatory AR) and compared to an antigen receptor having only intra-chain antigen binding (bivalent non-combinatory AR).

Figure 7 is a histogram showing cytotoxic efficacies of CAR Cl6-modified T-cells towards ovarian carcinoma cells Sk-ov-3. Tumor cells have been electroporated with increasing amounts of Cl6 RNA, CD8⁺ T cells have been electroporated with different antigen receptor expression constructs. T cell effector function as measured by cell lysis decreased with decreasing amounts of Cl6 expressed in the Sk-ov-3 cells. Cytotoxicity of the T cells recombinantly expressing bivalent antigen receptors (tandem AR and inter-combinatory AR)

was less dependent on antigen density on the target cells as compared to the monovalent non-combinatory antigen receptor and classical scCAR.

Figures 8A-8D are histograms showing the results of immune cell activation as determined by cell proliferation against iDCs as APC. Figures 8A and 8B: Proliferation of CFSE labeled CD4⁺ (Figure 8A) and CD8⁺ T cells (Figure 8B), respectively, against Cl6 negative immature dendritic cells (iDCs). Upon co-stimulation with molecules CD80 and 41BBL in cis (i.e. electroporation into T-cells), the classical single chain chimeric antigen receptor (classical scCAR) revealed background proliferation in the absence of its cognate antigen. This was also observed for T cells incubated without any target cells, indicating that non-specific proliferation is an intrinsic characteristic of T cells expressing the classical scCAR (data not shown). This result was also seen with the Cl6 negative cell line Sk-Ov-3. Figures 8C and 8D: Proliferation of CFSE labeled CD4⁺ (Figure 8C) and CD8⁺ T cells (Figure 8D), respectively, against Cl6⁺ iDCs. CD4⁺ T cells proliferated against Cl6 loaded iDCs when electroporated with a construct expressing the inter-combinatory AR. The classical scCAR design led to less proliferation. For CD8⁺ T cells both, the classical scCAR and inter-combinatory AR showed almost the same good proliferation.

Figures 9A-9B are histograms showing the results of immune cell activation as determined by cell proliferation against tumor cells as APC. Proliferation of CFSE labeled CD4⁺ (Figure 9A) and CD8⁺ (Figure 9B) T cells against the Cl6⁺ ovarian carcinoma cell line Ov-90 electroporated with costimulatory molecules CD80 and 41BBL (co-stimulation in trans). CD4⁺ T cells were able to proliferate against Ov-90^{Cl6+CD80+41BBL+} when electroporated with the inter-combinatory AR in analogy to iDC^{Cl6+}. Both the monovalent and bivalent antigen receptors led to proliferation of CD8⁺ T cells. The amount of proliferation was comparable with the classical scCAR. Co-stimulation of T cells in cis further enhanced T cell proliferation.

Figure 10 is a histogram showing the antigen dose-dependent induction of IFN- γ production. Detection of IFN- γ production in ELISA for CD8⁺ T cells electroporated with different antigen receptor expression constructs and controls, and co-cultured with immature dendritic cells (iDC) electroporated with relevant full-length Cl6 RNA in the range of 0.01 μ g – 1 μ g and 1 μ g irrelevant gp100. Bivalent antigen receptors were assessed for cytokine secretion dependent on the hypothesized amount of combinatory chain pairing with the order inter- > inter/intra- > bivalent non-combinatory AR. At high antigen density (1 μ g Cl6) the classical

CAR elicited the highest amount of IFN γ -secretion followed by inter- and inter/intra-combinatory AR. Bivalent non-combinatory AR was less functional than the monov. combinatory AR and a monovalent non-combinatory AR. Bivalent non-combinatory AR rely on chain pairing merely between the invariant human C-domains, while the monovalent combinatory AR accomplishes better chain pairing between the human C- and mouse V-domains. The monovalent non-combinatory AR is more functional than the bivalent non-combinatory AR due to the fact that inter-chain pairing is promoted by mouse C-domains instead of weaker human ones. This is also true for any antigen dosis applied in this assay. Notably, when lowering the antigen densities (0.1 μ g, 0.01 μ g of Cl6 RNA-electroporated cells) the inter-combinatory AR became increasingly more reactive towards iDCs RNA-pulsed with relevant antigen when compared with the classical CAR. This is in line with a similar trend shown in an antigen dose-dependent cytotoxicity assay (Figure 7) towards a Cl6-electroporated tumor cell line Skov-3.

Figure 11 is a histogram showing the antigen dose-dependent induction of IFN- γ production. In Figure 11 A, a comparison of the formats of the classical and the novel combinatory classical CAR is shown. Both CARs rely on signaling via the fused CD3 ζ -signaling moiety independent of the endogenous CD3 complex in T-cells. Figure 11 B shows the detection of IFN- γ production in ELISA for CD8 $^{+}$ T cells electroporated with the Claudin 6-specific classical CAR and the novel combinatory classical CAR, both carrying CH2-CH3-antibody domains and CD28, and CD3 ζ as signal transmission domain. They were co-cultured with immature dendritic cells (iDC) electroporated with relevant full-length Cl6 RNA in the range of 0.002 μ g – 2 μ g and 2 μ g irrelevant gp100. At higher antigen densities (2 μ g – 0.2 μ g Cl6) both CAR formats elicited equal high amounts of IFN γ -secretion. For lower antigen densities the classical CAR seemed to be somewhat more efficient in secreting IFN γ . Importantly, the combinatory classical CAR is functional in the same range of titrated antigen as the classical CAR.

EXAMPLES

The techniques and methods used herein are described herein or carried out in a manner known per se and as described, for example, in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, 2nd Edition (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. All

methods including the use of kits and reagents are carried out according to the manufacturers' information unless specifically indicated.

Example 1: Expression of antigen receptors in T cells

The expression of various antigen receptor constructs was assessed one day after electroporation into CD8⁺ T cells using a Cl6 scFv idiotype-specific antibody. The constructs or combination of constructs tested for expression were (i) the constant domain of the murine T cell receptor alpha chain alone (mC α); (ii) VH-VL-mC β alone (scFv-mC β); (iii) VH-VL-CH2-CH3-CD28-CD3 ζ (Classical scCAR); (iv) mC α and VH-VL-mC β (monovalent non-combinatory AR); (v) VH-VL-mC α and VH-VL-mC β (bivalent non-combinatory AR); (vi) VL-VH-mC α and VH-VL-mC β (Intra/Inter-combinatory AR); (vii) mC α and VH-VL-VH-VL-mC β (Tandem AR); (viii) VH-hC α and VL-hC β (monovalent combinatory AR); (ix) VH-(GGGGS)₃-VH-hC α and VL-(GGGGS)₃-VL-hC β (Inter-combinatory AR 3GS); and (x) VH-(GGGGS)₃-VH-mC α and VL-(GGGGS)₃-VL-mC β (Inter-combinatory AR Mu 3GS) ("m" or "Mu" indicates murine origin and "h" indicates human origin of the constant domain). These different antigen receptor constructs are schematically presented in Figures 4A-4B. The data in Figure 5 represent results from two separate measurements for the bivalent non-combinatory, the intra/inter-combinatory and monovalent combinatory antigen receptors; five measurements for the tandem and inter-combinatory antigen receptors; and up to ten measurements for the classical scCAR and monovalent non-combinatory antigen receptors. The MFI value of each sample was normalized to the corresponding negative control (mC α or scFv-mC β set to 1).

As shown in Figure 5, the classical scCAR design showed the best expression. The monovalent non-combinatory, bivalent non-combinatory, intra/inter-combinatory and the inter-combinatory antigen receptors with human (hC) or murine (mC) constant domains showed similar expression. The monovalent combinatory AR showed reduced surface staining, whereas the tandem antigen receptor showed increased surface staining, as compared to the other constructs, except for the classical scCAR.

Example 2: IFN- γ secretion assay

On day 1 of the experiment, fresh peripheral blood mononuclear cells ("PBMCs") were isolated from a buffy coat of one healthy donor. From $\frac{3}{4}$ of PBMCs, CD14⁺ cells were isolated using MACS sort. MACS flow through and residual PBMCs were then MACS sorted for CD8⁺ T

cells. CD14⁺ cells were differentiated towards immature dendritic cells ("iDCs") by administration of IL-4 & GM-CSF (1000U/ml) on day 1, 3, 6. CD8⁺ T cells were transferred on OKT3 coated 6 well plates. On day 3, T cells were transferred to new 6 well plates. On day 7, iDCs were electroporated with irrelevant and Cl6 IVT RNA. OKT3 activated T cells were subsequently electroporated with controls, or antigen receptor constructs as set forth in the individual figures and as described in Example 1. For quality assurance, antigen receptor surface expression on T cells was analyzed using a Cl6 antigen receptor idiotype-specific antibody labeled with Dylight 650 on day 8. The electroporated T cells and antigen electroporated iDCs were subsequently co-cultured in a 96 well plate for 20h at an E:T ratio of 10:1 in duplicates. On day 9, culture supernatants were taken and analyzed for the amount of secreted IFN- γ in a sandwich ELISA using the IFN- γ Ready Set Go! kit from eBioscience (#88-7316-88). Absorbance was detected using a Tecan Sunrise ELISA reader.

As depicted in Figure 6A, mock (mC α) electroporated T cells in culture with Cl6 positive or negative iDCs showed no non-specific IFN- γ production. Of the antigen receptor positive T cells only the classical scCAR positive cells produced a detectable background of the cytokine when cultured with Cl6 negative target cells. IFN- γ production from monovalent antigen receptor positive cells could be observed when cultured with Cl6⁺ iDCs. Here, the classical scCAR produced high amounts of IFN- γ . When electroporated with a negative control of only one chain of the combinatory antigen receptor, in this case VH-VH-C α , no IFN- γ production could be observed. This control proved that both chains are required for antigen recognition and subsequent activation of the T cells. In contrast, the inter-combinatory AR (3GS) showed a pronounced improvement in IFN- γ production when compared to that produced with the monovalent AR (combinatory and non-combinatory). Different linker lengths, 2 or 4 repeats of the Gly4Ser linker between the variable domains, showed no major impact on function, as compared to the 3 repeats.

Importantly, a truncation of the N-terminal variable domain in the combinatory antigen receptor structure showed a strong reduction in effector function. This observation clearly proved that bivalent antigen-binding supports T cell activation. The prominent increase in function between the monovalent combinatory AR and the bivalent combinatory AR is also a strong indication that, besides the improvement of chain-pairing mediated by the inter-chain variable domains, the incidence of antigen binding itself across the two chains further stabilizes receptor chain pairing and hence, improves incorporation into the endogenous CD3 complex and subsequent T cell

activation/function. From double chain T cell receptors it is well-known that heterodimerisation of the chains is an essential prerequisite for efficient incorporation into the CD3 complex.

The integration of murine residues into the combinatory AR structure was able to further increase activation, which is reasoned to be due to the known stronger interaction of murine TCR constant $C\alpha/\beta$ -domains as compared to human ones. Thus, improved heterodimerisation of the peptide chains of the antigen receptor, either through inter-chain antigen binding to the variable domains or through dimerization of the T cell receptor constant domains on the individual chains, improved integration of the antigen receptor into the endogenous CD3 complex, and thus, improved T cell function. These results were highly reproducible with a different T cell donor (see Figure 6B).

Notably, the classical scCAR demonstrated in this experiment a non-specific background of IFN- γ production against Cl6 negative iDCs. This result is also reproducible and is in line with data published by Long et al. ((2015) Nat. Med., (21) 581-590) discussing tonic signaling by the classical scCAR-CD28-CD3 ζ fusion format. Long et al. observed an antigen-independent activation for several classical scCARs of varying antigen specificities. It is assumed that non-specific background activation of classical scCAR positive T cells is not a T cell donor specific effect.

Further, and as depicted in Figure 6C, bivalent non-combinatory and intra/inter-combinatory antigen receptors were tested together with the tandem bivalent antigen receptor for IFN- γ production. For comparison, the negative control, the monovalent non-combinatory AR, and as a positive control, the classical scCAR are also shown in the figure. The tandem bivalent antigen receptor showed an improvement compared to the monovalent antigen receptor. The bivalent non-combinatory antigen receptor VH-VL- $C\alpha$ + VH-VL- $C\beta$ elicited lower IFN- γ production as compared to the related intra/inter-combinatory antigen receptor VL-VH- $C\alpha$ + VH-VL- $C\beta$. These observations confirmed the hypothesis that inter-chain interactions conferred by both, V-domain pairing and antigen binding to variable domains on different chains stabilizes the heterodimeric topology and provides for a more robust receptor-dependent T cell signaling response.

Example 3: Cytotoxicity assay

On day 1 of the experiment, fresh PBMCs were isolated from two buffy coats from two healthy donors. PBMCs were MACS sorted for CD8⁺ T cells. CD8⁺ T cells were transferred on OKT3 coated 6 well plates. They were cultured in medium containing 50U/ml IL-2. On day 3, T cells were transferred on new 6 well plates and culture medium was changed. On day 7, the ovarian carcinoma cell line Sk-Ov-3 was RNA-electroporated with varying amounts of Cl6 RNA and 10µg of luciferase RNA. OKT3 activated T cells were electroporated with an irrelevant classical scCAR, a relevant Cl6-specific classical scCAR, a monovalent non-combinatory antigen receptor and a tandem antigen receptor as well as an inter-combinatory antigen receptor of the invention as indicated in Figure 7 and described above in Example 1. For quality assurance, antigen receptor surface expression on T cells was analyzed using a Cl6 antigen receptor idotype specific antibody labeled with Dylight 650 on day 8. The antigen receptor electroporated T cells and antigen electroporated Sk-Ov-3 were subsequently co-cultured in a 96 well plate for 3h at an E:T ratio of 30:1 in triplicates. After 3h of incubation, luciferin was added to each culture. Specific lysis was detected by a decrease in luciferin-signal due to its turnover by released luciferase in the TECAN reader.

The Sk-Ov-3 data impressively document that the bivalent antigen receptors (inter-combinatory AR and tandem AR) of the invention showed a marked improvement as compared to the classical scCAR and monovalent non-combinatory AR. Compared to all other antigen receptor constructs, the classical scCAR design showed the best lysis of about 77% against Cl6 electroporated Sk-Ov-3 cells. However, it should be mentioned that 10µg of antigen RNA is a non-physiological condition and does not accurately reflect the *in vivo* situation. At low antigen doses, the monovalent non-combinatory AR was not able to lyse tumor cells in a satisfactory fashion (9.2%). In contrast, the inter-combinatory AR (3GS) still showed good specific lysis (41.3%) compared to the classical scCAR design (48.1%) and conclusively, was less dependent on antigen density for substantial cytotoxic effector function.

Example 4: Proliferation assay

On day 1 of the experiment, fresh PBMCs were isolated from a buffy coat of a healthy donor. From ¾ of PBMCs, CD14⁺ cells were isolated using MACS sort, and residual PBMCs were frozen. CD14⁺ cells were differentiated to iDCs by administration of IL-4 & GM-CSF

(1000U/ml) on day 1, 3, 6. On day 7 iDCs were electroporated with irrelevant and Cl6 IVT-RNA. The frozen PBMCs were thawed on the same day and MACS sorted for CD4⁺ and CD8⁺ cells. Without any prior activation (OKT3), naïve T cells, 6 and 7x10⁶ cells, respectively, were subsequently electroporated with controls, classical, monovalent and bivalent antigen receptor constructs as indicated in Figures 8A-8D, which constructs were described in Example 1. In an independent set of T cell responders, the same antigen receptor constructs were also electroporated along with the co-stimulatory molecules 41BBL and CD80 to achieve an auto-co-stimulation, or co-stimulation in cis, which was demonstrated to improve effector function.

For quality assurance, antigen receptor and 41BBL + CD80 expression on T cells was analyzed by FACS staining on day 8. T cells were subsequently labeled with the proliferation marker CFSE. The electroporated T cells and iDCs as well as the ovarian carcinoma cell line OV-90 were subsequently co-cultured in a 96 well plate for 5 days at an E:T ratio of 10:1 in duplicates. On day 5, cultured cells were stained in the 96 well plates with CD4 or CD8 antibodies labeled with APC-Cy7. Proliferation of T cells was detected via FACS by the reduction of CFSE-signal due to dilution in proliferating daughter cells. With minor adjustments, daughter population sizes were assessed using the in Flowjo implemented proliferation tool. The sum of proliferating cells is depicted as daughter generations.

Background proliferation of T cells was assessed for cells cultured with either the Cl6 negative cell line Sk-Ov-3 and Cl6 negative iDCs. Independently of the electroporated antigen receptor construct, neither CD4⁺ nor CD8⁺ T cells proliferated against Cl6 negative cells. Upon co-stimulation in cis, only classical scCAR engineered T cells proliferated non-specifically against Cl6 negative iDCs and Sk-Ov-3 (Sk-Ov-3 data not shown). CD4⁺ T cells proliferated overall less efficiently against Cl6⁺ cells compared to CD8⁺ T cells (see Figures 8C to 8D). This was in particular true when using Cl6⁺ Ov-90 tumor cells as target cells (Figures 9A to 9B).

The results for T cells co-cultured with iDCs proved the overall good function of the inter-combinatory antigen receptor. Of CD4⁺ T cells without co-stimulation in cis, the inter-combinatory antigen receptor showed superior proliferation and even outperformed the classical scCAR design. This effect was not due to a reduced surface expression of the classical scCAR, as analyzed by CAR idiotype staining. For CD8⁺ T cells, the inter-combinatory antigen receptor design demonstrated a remarkable proliferation against Cl6 loaded iDCs (70%). Co-stimulation of the T cells in cis could further enhance T cell responses.

Interestingly no proliferation of cells could be observed against the Cl6+ ovarian carcinoma cell line OV-90 (data not shown). This was reasoned to be due to a lack of co-stimulatory molecules on the surface of the tumor cells. In order to compensate this lack of co-stimulus, Ov-90 cells were electroporated with CD80 and 41BBL RNA. In this case, proliferation of CD4+ and CD8+ T cells could be detected (see Figures 9A and 9B). Notably, for CD4+ cells this was only the case for the inter-combinatory AR design (15%). For CD8+ cells, the proliferation pattern looked different. The monovalent non-combinatory AR, classical scCAR and inter-combinatory AR responses were comparable with 60% proliferating cells. Upon co-stimulation in cis, proliferation of in particular CD4+ T cells was improved.

The data clearly indicate that the bivalent antigen receptor construct (Inter-combinatory AR) is capable of proliferating against Cl6+ tumor cell lines, when co-stimulated. In general, the inter-combinatory AR is by far better than the monovalent non-combinatory AR in proliferating against iDCs loaded with the cognate target antigen. The classical scCAR is prone to non-specific responses when co-stimulated in cis against Cl6 negative iDCs, indicating a higher susceptibility for antigen-independent T cell signaling.

Example 5: Antigen-titrated IFN γ - secretion assay

On day 1 of the experiment, fresh peripheral blood mononuclear cells ("PBMCs") were isolated from a buffy coat of one healthy donor. From $\frac{3}{4}$ of PBMCs, CD14+ cells were isolated using MACS sort. MACS flow through and residual PBMCs were then MACS sorted for CD8+ T cells. CD14+ cells were differentiated towards immature dendritic cells ("iDCs") by administration of IL-4 & GM-CSF (1000U/ml) on day 1, 3, 6. CD8+ T cells were transferred on OKT3 coated 6 well plates. On day 3, T cells were transferred to new 6 well plates. On day 7, iDCs were electroporated with irrelevant and Cl6 IVT RNA dose-dependently. OKT3 activated T cells were subsequently electroporated with controls, or antigen receptor constructs as set forth in the individual figures and as described in Example 1. For quality assurance, antigen receptor surface expression on T cells was analyzed using a Cl6 antigen receptor idiotype-specific antibody labeled with Dylight 650 on day 8. The electroporated T cells and antigen electroporated iDCs were subsequently co-cultured in a 96 well plate for 20h at an E:T ratio of 10:1 in duplicates. On day 9, culture supernatants were taken and analyzed for the amount of secreted IFN- γ in a sandwich ELISA using the IFN- γ Ready Set Go! kit from eBioscience (#88-

7316-88). Absorbance was detected using a Tecan Sunrise ELISA reader. The result are shown in Figure 10.

Bivalent antigen receptors were assessed for the amount of cytokine secretion dependent on the hypothesized propensity to pair in a combinatory fashion which favours stable expression and subsequently, T cell signaling: We speculated to observe exclusive combinatory V-domain inter-chain pairing for the inter-combinatory AR while the inter/intra-combinatory AR may coexist in a proportion of less favourable intra-chain pairing. At high antigen density (1 μg Cl6) the classical CAR elicited the highest amount of IFN γ -secretion followed, as expected, by inter- and inter/intra-combinatory AR. Bivalent non-combinatory AR was less functional than the references of a monovalent combinatory AR and a monovalent non-combinatory AR. Bivalent non-combinatory AR rely on chain pairing merely between the invariant human C-domains, while the monovalent combinatory AR accomplishes better chain pairing between the human C- and mouse V-domains. In line with the expected results, also the monovalent non-combinatory AR is more functional than the bivalent non-combinatory AR because inter-chain pairing, despite the fact that it is also restricted to C-domains here, is promoted by mouse C-domains instead of weaker human ones. This is also true for any antigen dosis applied in this assay.

Notably, when lowering the antigen densities (0.1 μg , 0.01 μg of Cl6 RNA-electroporated cells) the inter-combinatory AR became increasingly more reactive towards iDCs pulsed with relevant RNA when compared with the classical CAR. This is in line with a similar trend shown in a antigen dose-dependent cytotoxicity assay (Figure 7) towards a Cl6-electroporated tumor cell line Skov-3.

The classical CAR was also compared with a novel combinatory classical AR in an antigen-titrated IFN γ -secretion assay (Figure 11). Both chimeric antigen receptors comprise CH2-CH3-antibody domains and CD28, and CD3 ζ as signal transmission domain. They were co-cultured with immature dendritic cells (iDC) electroporated with relevant full-length Cl6 RNA in the range of 0.002 μg – 2 μg and 2 μg irrelevant gp100. At higher antigen densities (2 μg – 0.2 μg Cl6) both CAR formats elicited equal high amounts of IFN γ -secretion. For lower antigen densities the classical CAR seemed to be somewhat more efficient in secreting IFN γ . Importantly, the combinatory classical CAR is functional in the same range of titrated antigen as the classical CAR down to very low amounts of endogenously expressed Claudin 6. However, this format of a combinatory AR, as opposed to the TCR C α /C β -based combinatory AR design,

apparently leads to equal but not better T-cell signaling than the classical CAR presumably due to exogenous CD3 ζ -dependent but endogenous CD3-independent signaling. The former mechanism is supposed to be independent of endogenous CD3-recruitment to the TCR or CAR, respectively, which in turn would regulate the amount of T-cell activation.

WE CLAIM:

1. An antigen receptor, which receptor comprises a first peptide chain and a second peptide chain, wherein

the first peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain;

the second peptide chain comprises at least a first and a second domain, and an immunoreceptor signal transmission domain;

wherein the immunoreceptor signal transmission domain is preferably of human origin,

wherein the first domain from the first peptide chain forms together with one of the domains from the second peptide chain a first antigen binding site, and

wherein the second domain from the first peptide chain forms together with the other domain from the second peptide chain a second antigen binding site.

2. The receptor of claim 1, wherein the immunoreceptor signal transmission domain comprises a constant or invariant region of a T cell receptor chain or a constant or invariant region of an immune cell Fc receptor chain or a portion of the constant or invariant region.

3. The receptor of claim 1 or 2, wherein the first and/or second peptide chains further comprise a linker between the first and second domains and/or between the first and second domains and the immunoreceptor signal transmission domain.

4. The receptor of any one of claims 1 to 3, wherein the first and/or second domains each comprise a variable region of an immunoglobulin chain or a variable region of a T cell receptor chain or a portion of the variable region.

5. The receptor of any one of claims 1 to 4, wherein

(i) the first peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof, or

(ii) the first peptide chain comprises a constant region of a T cell receptor beta chain or a portion thereof and the second peptide chain comprises a constant region of a T cell receptor alpha chain or a portion thereof.

6. The receptor of any one of claims 1 to 5, wherein the first domain from the first peptide chain comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the domain from the second peptide chain forming an antigen binding site with the first domain from the first peptide chain comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof.

7. The receptor of any one of claims 1 to 6, wherein the second domain from the first peptide chain comprises a variable region of a heavy chain of an immunoglobulin with a specificity for an antigen or a portion thereof and the domain from the second peptide chain forming an antigen binding site with the second domain from the first peptide chain comprises a variable region of a light chain of an immunoglobulin with a specificity for the antigen or a portion thereof.

8. The receptor of any one of claims 1 to 7, wherein the first and second antigen binding sites bind to the same antigen or different antigens.

9. The receptor of any one of claims 1 to 8, wherein
the first and the second domains from the first peptide chain each comprise a variable region of a heavy chain of an immunoglobulin or a portion thereof; and
the first and the second domains from the second peptide chain each comprise a variable region of a light chain of an immunoglobulin or a portion thereof.

10. The receptor of any one of claims 1 to 9, wherein
the N-terminal domain from the first peptide chain forms together with the N-terminal domain from the second peptide chain an antigen binding site; and
the C-terminal domain from the first peptide chain forms together with the C-terminal domain from the second peptide chain an antigen binding site.

11. A peptide chain comprising a first and a second domain which each comprise a variable region of a heavy chain of an immunoglobulin or each comprise a variable region of a light chain of an immunoglobulin and wherein the peptide chain further comprises an immunoreceptor signal transmission domain.

12. A recombinant cell expressing both the first and second peptide chains defined in any one of claims 1 to 10.

13. A recombinant cell expressing the peptide chain of claim 11.

14. An *ex vivo* method for producing a cell expressing an antigen receptor which receptor comprises a first peptide chain and a second peptide chain, the method comprising:

- (a) providing a cell, preferably a human cell;
 - (b) providing a first genetic construct encoding the first peptide chain comprising at least a first and a second domain, and an immunoreceptor signal transmission domain;
 - (c) providing a second genetic construct encoding the second peptide chain comprising at least a first and a second domain and an immunoreceptor signal transmission domain;
 - (d) introducing the first and second genetic constructs into the cell; and
 - (e) allowing the constructs to be expressed in the cell;
- wherein the cell is preferably a T cell,
- wherein the first domain from the first peptide chain is able to form together with one of the domains from the second peptide chain a first antigen binding site, and
- wherein the second domain from the first peptide chain is able to form together with the other domain from the second peptide chain a second antigen binding site,
- wherein the first peptide chain and the second peptide chain are preferably provided on a single genetic construct.

15. A nucleic acid encoding both the first and second peptide chains defined in any one of claims 1 to 10, wherein the nucleic acid is preferably DNA or RNA.

16. A nucleic acid encoding the peptide chain of claim 11, wherein the nucleic acid is preferably DNA or RNA.

17. A pharmaceutical composition comprising

- (a) the antigen receptor of any one of claims 1 to 10,
- (b) the recombinant cell of claim 12,
- (c) a nucleic acid of claim 15, or
- (d) a nucleic acid encoding the first peptide chain *and* a nucleic acid encoding the second peptide chain, said chains being defined as in any one of claims 1 to 10;

and a pharmaceutically acceptable carrier, for use as a medicament.

18. Use of the antigen receptor of any one of claims 1 to 10, the recombinant cell of claim 12, or the nucleic acid of claim 15; and a pharmaceutically acceptable carrier in the manufacture of a pharmaceutical composition for treating cancer.