High affinity T cell receptor and use thereof

FIELD OF THE INVENTION

The present invention is directed to a high affinity T cell receptor (TCR) against a tumor-associated antigen, an isolated nucleic acid molecule encoding same, a T cell expressing said TCR, and a pharmaceutical composition for use in the treatment of diseases involving malignant cells expressing said tumor-associated antigen.

BACKGROUND OF THE INVENTION

TCR's are members of the immunoglobulin superfamily and usually consist of two subunits, namely the α- and β-subunits. These possess one N-terminal immunoglobulin (Ig)-variable (V) domain, one Ig-constant (C) domain, a transmembrane/cell membrane-spanning region, and a short cytoplasmic tail at the C-terminal end. The variable domains 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 alpha 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 constant domain of the TCR domain consists of short connecting sequences in which a cysteine residue forms disulfide bonds, which forms a link between the two chains.

The affinity of TCR's for a specific antigen makes them valuable for several therapeutic approaches. For example, cancer patients, such as melanoma patients, can be effectively treated by using adoptive immunotherapy.

The adoptive transfer of lymphocytes in the setting of allogeneic stem cell transplantation (SCT) has demonstrated the power of the immune system for eradicating hematological malignancies (Kolb et al. 1995). It appears that SCT can also function to eliminate solid tumors, such as renal cell carcinomas (RCC) in some cases (reviewed in Kolb et al. 2004 and Dudley and Rosenberg, 2003). In SCT recipients, the elimination of malignant cells may only occur after several months up to a year, due to the fact that specific T cells must be activated *in vivo* and must then expand to adequate numbers following the development of the new hematopoietic system in the transplant recipient. Alternatively, after a period of time (approximately 60 days) during which tolerance is established in the SCT recipient, a transfer of unprimed, unseparated lymphocytes can be made to speed up the generation of immune responses directed against tumor cells. Here again, the specific lymphocytes capable of attacking tumor cells must be activated and expanded from the low frequency precursor lymphocytes that are present among the unselected population of lymphocytes that are transferred. Donor lymphocyte infusions (DLI) of unselected lymphocyte populations after SCT work well for the elimination of chronic myelogenous leukemia (CML), which grows slowly, but are less effective in the eradication of acute leukemia, partly due to the fact that the growth of the malignant cells outpaces the expansion capacity of the immune cells. This same expansion differential in which immune cells expand more slowly than tumor cells, also impacts on the poor immune elimination of rapidly progressing solid tumors. A second handicap in the use of unselected mixed lymphocyte populations in DLI is that T cells may also be transferred that have the capacity to attack normal cells and tissues of the recipient, leading to graft-versus-host-disease (GVHD), a disease with high morbidity and mortality.

Recent studies have demonstrated that the adoptive transfer of selected T cells with defined peptide specificities can lead to major reductions in tumor burden in an autologous setting, particularly if patients have been pretreated with non-myeloablative regimens (Dudley et al. 2002, 2003). This eliminates the need to perform SCT in the tumor patient, and thereby also bypasses the problem of GVHD.

In order to extend the capacity to use adoptive cell therapy (ACT) to treat patients with more rapidly growing tumors, it is a goal to transfer enriched, peptide-specific effector T cells (both CD4 T helper cells and cytotoxic T lymphocytes) that have been selected for their ligand specificities to effectively attack tumor cells while avoiding serious attack of normal tissues. These cells are to be rapidly expanded to large numbers *ex vivo* and then used for ACT. Alternatively, the T cell receptors (TCR) of such ligand-specific T cells can be cloned and expressed as TCR-transgenes in activated lymphocytes, using either recipient peripheral blood lymphocytes or activated T cell clones with defined specificities that grow well and do not have the capacity to attack normal host tissues.

As examples, an expanded allospecific T cell clone that is specific for an MHC molecule not expressed by the recipient or an expanded T cell clone specific for a virus, such as cytomegalovirus or Epstein-Barr virus, could be used as recipient cells for the transgenic TCR. The availability of a panel of transgenic TCR vectors, recognizing different MHC-peptide ligands could be used to develop large numbers of pre-activated T cells of both the CD4 and CD8 subtypes, thereby allowing large numbers of effector lymphocytes to be rapidly prepared and transferred to patients whose tumors express the corresponding TCR ligands. This would save time in achieving the numbers of specific T cells required to control tumor growth, possibly leading to more effective tumor eradication of rapidly progressing tumors.

Because the determinants that specific T cells recognize on leukemia and lymphomas, as well as solid tumor cells, often represent self-peptides derived from over-expressed proteins that are presented by self-MHC molecules, the affinity of their T cell receptors (TCR) is low, since T cells bearing high affinity receptors have been eliminated through the process of negative selection which is applied to lymphocytes during their development in the thymus to prevent autoimmunity. More effective tumor cell recognition occurs if the T cells are generated from lymphocyte populations that have not been negatively selected against self-MHC-molecules during their development in the thymus.

WO 2006/031221 pertains to T cell receptors against tumor-associated antigens, nucleic acids encoding the same, vectors and cells comprising the nucleic acids encoding the T cell receptors, and methods of use thereof. Among others, it is disclosed that the TCR subunits

have the ability to form TCR that confer specificity to T cells for tumor cells presenting MART-I, NY-ESO-I, and melanoma-related gp100.

In the prior art, several scientific and patent documents are existing, which describe TCR, which are able to recognise and bind tyrosinase. Visseren et al. (Int. J. Cancer (1997) 72, 1122-1128) describe the affinity and specificity of several tyrosinase-specific TCR and suggest to use these TCR as a specific treatment of melanoma patients.

Roszkowski et al. (J. Immunol. (2003) 170, 2582-2589 and Cancer Res. (2005) 65, 1570-1576) the like are characterising tyrosinase-specific TCR.

US 5,906,936 is directed to cytotoxic T-cells which kill non-MHC-restricted target cells and not to cells, which comprise specific TCR sequences.

WO97/32603 is directed to a method for producing non-human TCR and TCR specific for human HLA-restricted tumor antigens. Furthermore, the TCR-nucleic acids and recombinant T-cells are described as well as the administration of TCR recombinant T-cells for the treatment of several diseases.

WO2007/065957 describes an effector T-cell transfected with an antigen specific TCR coding RNA wherein the transfected T-cell recognizes the antigen in a complex with the MHC-molecule and binds the same. As a potential tumor antigen, MART-1 (Melan-A), tyrosinase and survivin are named.

WO2008/039818 discloses MART-1 and tyrosinase-specific TCR sequences and describes the enhancement of antigen recognition by substitution in the CDR2 region.

The above prior art TCR sequences are all derived from autologous or xenogeneic, but not allogeneic, sources.

For example, TCR sequences are from peripheral blood or from tumor infiltrating lymphocytes of HLA-A2 positive melanoma patients. This means that all these TCR are HLA-A2 self-restricted TCRs, or, are HLA-DP4 restricted, NY-ESO-1 specific, both

derived from autologous sources. As an alternative, as disclosed in WO97/32603, the TCR is derived from an HLA-A2 transgenic mouse, so in this case the sequence is xenogeneic. However, the available prior art documents do not show TCR sequences, which are allo-restricted and tyrosinase-specific.

Thus, there is still an important need to find means to generate T cells that bear TCR with high functional avidity that have the capacity to recognize specific ligands on tumor cells. Although adoptive transfer of T cells expressing transgenic T cell receptors (TCR) with anti-tumor function is a hopeful new therapy for patients with advanced tumors, there is a critical bottleneck in identifying high-avidity T cells with TCR specificities needed to treat different malignancies.

SUMMARY OF THE INVENTION

Therefore, it is an object of the present invention to provide TCR or functional parts thereof, such as CDR3 regions, which show high affinity against tumor-associated antigens, in particular tyrosinase. It is a further object of the invention to provide pharmaceutical compositions for use in adoptive cell therapy which allow an effective treatment of diseases involving malignant cells expressing tyrosinase, preferably melanomas, gliomas, glioblastomas, and/or rare tumors of ectodermal origin.

These objects are solved by the subject-matter of the independent claims. Preferred embodiments are indicated in the dependent claims.

TCR specific for the melanoma-associated antigen, tyrosinase, could be isolated by the inventors and it could be shown that TCR derived from the allo-restricted clone were superior in recognition of specific peptide and tumor cells after expression as transgenes in recipient lymphocytes. Therefore, TCR's and functional parts thereof, such as CDR3 regions could be identified, which find application in adoptive cell therapy for the treatment of several malignancies.

A number of T cell clones with specificity for various tumor-associated antigens have been reported over the years (see above). Most of these TCR are restricted by self-MHC molecules. The TCR sequences disclosed herein, however, are allo-restricted and show

high-avidity in recognition of their specific ligands. The TCR of the present invention are not self-MHC-restricted and therefore have higher structural affinity for interactions with MHC-peptide ligands that target tumor cells via common over-expressed self proteins. As it will be outlined in the Examples, the TCR of the present invention were derived from a T cell clone generated by priming CD8* T cells with autologous dendritic cells from an HLA-A2 negative donor co-expressing allogeneic HLA-*A0201 molecules and an antigen. As a result, the present TCR are of therapeutic use for the treatment of HLA-A2 positive patients.

In more detail, T cell responses against tumors are often directed against self-MHC molecules presenting peptides derived from over-expressed self-proteins. In general, T cells with high avidity for self-peptide/self-MHC ligands are eliminated by negative selection to prevent autoimmunity. The TCR affinity of remaining T cells specific for self-ligands is normally low, however high-avidity T cells are needed to effectively eradicate tumors. Because negative selection is limited to self-MHC molecules, T cells that recognize allogeneic MHC molecules have not undergone negative selection. However, as described in the present invention if peptides are presented by allogeneic MHC molecules, it is feasible to obtain high-avidity T cells specific for common tumor-associated ligands derived from over-expressed self-proteins.

DETAILED DESCRIPTION OF THE INVENTION

According to a first aspect, the present invention provides a nucleic acid molecule coding for the V(D)J regions of a TCR that recognizes a tumor antigen and comprising the nucleic acid sequence of SEQ ID NO: 1 coding for the α-chain and/or comprising the nucleic acid sequence of SEQ ID NO: 2 coding for the β-chain of said TCR.

Therefore, a TCR of the present invention and a nucleic acid sequence encoding the same may comprise only one of the α-chain or β-chain sequences as defined herein (in combination with a further α-chain or β-chain, respectively) or may comprise both chains.

The term "nucleic acid" as used herein with reference to nucleic acids refers to a naturally-occurring nucleic acid that is not immediately contiguous with both of the sequences with which it is immediately contiguous (one on the 5'end and one on the 3'end) in the naturally-

occurring genome of the cell from which it is derived. For example, a nucleic acid can be, without limitation, a recombinant DNA molecule of any length, provided one of the nucleic acid sequences normally found immediately flanking that recombinant DNA molecule in a naturally-occurring genome is removed or absent. Thus, a nucleic acid includes, without limitation, a recombinant DNA that exists as a separate molecule (e. g., a cDNA or a genomic DNA fragment produced by PCR or restriction endonuclease treatment) independent of other sequences as well as recombinant DNA that is incorporated into a vector, an autonomously replicating plasmid, a virus (e. g., a retrovirus, or adenovirus). In addition, an isolated nucleic acid can include a recombinant DNA molecule that is part of a hybrid or fusion nucleic acid sequence.

Furthermore, the term "nucleic acid" as used herein also includes artificially produced DNA or RNA sequences, such as those sequences generated by DNA synthesis based on *in silico* information.

The nucleic acids of the invention can comprise natural nucleotides, modified nucleotides, analogs of nucleotides, or mixtures of the foregoing as long as they are capable of causing the expression of a polypeptide *in vitro*, and preferably, in a T cell. The nucleic acids of the invention are preferably RNA, and more preferably DNA.

Furthermore, the present invention also comprises derivatives of the above described nucleic acid molecules, wherein, related to the above SEQ ID NO: 1 and 2, the sequence has been altered by additions, deletions and/or substitutions and wherein the tumor antigen recognizing characteristics are maintained or improved. In other words, the tumor antigen recognizing characteristics are at least maintained.

More precisely, such a derivative is coding for the α- or β-chain, wherein the chain has been altered by one or more additions or deletions of from 1-15 amino acids, the additions or deletions being outside the CDR3 region of each chain, and/or by conservative substitutions of from 1-15 amino acids. It is noted in this connection that also the CDR3 region may be altered, but to a lesser extent. The definition of those amendments is indicated below for the derivatives of fragments coding for the CDR3 region.

Useful changes in the overall nucleic acid sequence in particular are related to codon optimization and the addition of epitope tags, which will be explained in detail below. Such codon optimization can include optimization of expression levels, optimization of avidity for target cells, or both.

In general, it should, however, be noted that the alterations should not diminish or alter the ability of the encoded polypeptide to form part of a TCR that recognizes tumor associated antigens in the context of an MHC, but should facilitate destruction of a cancer cell, and preferably facilitate the regression of a tumor, or other cancerous state.

For example, alterations can be made which lead to conservative substitutions within the expressed amino acid sequence. These variations can be made in complementarity determining and non-complementarity determining regions of the amino acid sequence of the TCR chain that do not affect function. However, as noted above, additions and deletions should not be performed in the CDR3 region (for example an addition of epitope tags).

The concept of "conservative amino acid substitutions" is understood by the skilled artisan, and preferably means that codons encoding positively-charged residues (H, K, and R) are substituted with codons encoding positively-charged residues, codons encoding negatively-charged residues (D and E) are substituted with codons encoding negatively-charged residues, codons encoding neutral polar residues (C, G, N, Q, S, T, and Y) are substituted with codons encoding neutral polar residues, and codons encoding neutral non-polar residues (A, F, I, L, M, P, V, and W) are substituted with codons encoding neutral non-polar residues. These variations can spontaneously occur, be introduced by random mutagenesis, or can be introduced by directed mutagenesis. Those changes can be made without destroying the essential characteristics of these polypeptides, which are to recognize antitumor antigens in the context of an MHC with high avidity so as to enable the destruction of cancer cells. The ordinarily skilled artisan can readily and routinely screen variant amino acids and/or the nucleic acids encoding them to determine if these variations substantially lessen or destroy the ligand binding capacity by methods known in the art.

In a further embodiment, the present invention provides fragments of the above nucleic acid molecules, coding for a CDR3 region of a TCR recognizing a tumor antigen and having the nucleic acid sequence of SEQ ID NO: 3 or 4 or coding for the amino acid sequences of SEQ

ID NO: 5 or 6. Alterations in the CDR3 region will be performed according to the considerations described below.

The invention further provides derivatives wherein the CDR3 region has been altered by one or more additions and/or deletions of an overall number of from 1-5 amino acids, but not more than 1-3 contiguous amino acids and/or conservative substitutions of from 1-6 amino acids and wherein the tumor antigen recognizing characteristics are maintained or improved.

This means, more precisely, that additions or deletions may be performed to an extent that 1-5 amino acids are added or deleted in the CDR3 region. If more then one addition or deletion is performed, the overall number of added or deleted amino acids may not exceed 5 amino acids. Further, one single addition or deletion at one site may only be in the range of 1-3 amino acids, i.e. 1-3 contiguous amino acids, since the ligand binding capacity might be deteriorated by performing larger additions/deletions.

A preferred derivative of the nucleic acid molecule encoding the α- or β-chain of said TCR is one, wherein the original sequence of SEQ ID NO: 1 and 2 has been altered by codon optimization. A preferred example of such a derivative coding for the V(D)J regions of a TCR that recognizes a tumor antigen is the nucleic acid sequence of SEQ ID NO: 7 coding for the α-chain and the nucleic acid sequence of SEQ ID NO: 8 coding for the β-chain of said TCR.

Codon optimization is a generic technique to achieve optimal expression of a foreign gene in a cell system. Selection of optimum codons depends on codon usage of the host genome and the presence of several desirable and undesirable sequence motifs. It is noted that codon optimization will not lead to an altered amino acid sequence and, thus, will not fall under the definition of a conservative substitution as contained in this application.

In a preferred embodiment, the tumor antigen is tyrosinase. Tyrosinase expressing malignancies still have a high incidence, for example, around 160,000 new cases of melanoma are diagnosed worldwide each year. According to a report issued by WHO, about 48,000 melanoma related deaths occur worldwide per year. Thus, tyrosinase is a suitable tumor antigen which can serve as a target for tumor treatment.

In a second aspect, the present invention is directed to a TCR, preferably a soluble TCR, encoded by a nucleic acid molecule as defined above or comprising the amino acid sequences of SEQ ID NO: 5 and/or 6.

Said TCR preferably is present in the form of a functional TCR α-and/or β-chain fusion protein, comprising:

a) at least one epitope-tag, and

b) the amino acid sequence of an α and/or β chain of a TCR as defined above or encoded by a nucleic acid molecule as outlined above,

wherein said epitope-tag is selected from

i) an epitope-tag added to the N- and/or C-terminus of said α- and/or β-chain, or added into the α- and/or β-chain sequence, but outside the CDR3 region,

ii) an epitope-tag inserted into a constant region of said α- and/or β-chain, and

iii) an epitope-tag replacing a number of amino acids in a constant region of said α-and/or β-chain.

Epitope tags are short stretches of amino acids to which a specific antibody can be raised, which in some embodiments allows one to specifically identify and track the tagged protein that has been added to a living organism or to cultured cells. Detection of the tagged molecule can be achieved using a number of different techniques. Examples of such techniques include: immunohistochemistry, immunoprecipitation, flow cytometry, immunofluorescence microscopy, ELISA, immunoblotting ("Western"), and affinity chromatography. Epitope tags add a known epitope (antibody binding site) on the subject protein, to provide binding of a known and often high-affinity antibody, and thereby allowing one to specifically identify and track the tagged protein that has been added to a living organism or to cultured cells.

In the context of the present invention, a "functional" T-cell receptor (TCR) α- and/or β-chain fusion protein shall mean an α- and/or β-chain fusion protein that, although the chain includes the epitope-tag and/or has a tag attached to it, maintains at least substantial fusion protein biological activity in the fusion. In the case of the α- and/or β-chain of a TCR, this shall mean that both chains remain able to form a T-cell receptor (either with a non-modified α- and/or β-chain or with another inventive fusion protein α- and/or β-chain) which exerts its biological function, in particular binding to the specific peptide-MHC complex of said TCR, and/or functional signal transduction upon peptide activation.

Preferred is a functional T-cell receptor (TCR) α- and/or β-chain fusion protein according to the present invention, wherein said epitope-tag has a length of between 6 to 15 amino acids, preferably 9 to 11 amino acids.

Even more preferred is a functional T-cell receptor (TCR) α- and/or β-chain fusion protein according to the present invention, wherein said T-cell receptor (TCR) α- and/or β-chain fusion protein comprises two or more epitope-tags, either spaced apart or directly in tandem. Embodiments of the fusion protein can contain 2, 3, 4, 5 or even more epitope-tags, as long as the fusion protein maintains its biological activity/activities ("functional").

Preferred is a functional T-cell receptor (TCR) α- and/or β-chain fusion protein according to the present invention, wherein said epitope-tag is selected from, but not limited to, CD20 or Her2/neu tags, or other conventional tags such as a myc-tag, FLAG-tag, T7-tag, HA (hemagglutinin)-tag, His-tag, S-tag, GST-tag, or GFP-tag. myc, T7, GST, GFP tags are epitopes derived from existing molecules. In contrast, FLAG is a synthetic epitope tag designed for high antigenicity (see, e.g., U.S. Pat. Nos. 4,703,004 and 4,851,341). The myc tag can preferably be used because high quality reagents are available to be used for its detection. Epitope tags can of course have one or more additional functions, beyond recognition by an antibody. The sequences of these tags are described in the literature and well known to the person of skill in art.

In the functional T-cell receptor (TCR) α- and/or β-chain fusion protein according to the present invention, said fusion protein may be for example selected from two myc-tag sequences that are attached to the N-terminus of an α-TCR-chain and/or 10 amino acids of a protruding loop region in the β-chain constant domain being exchanged for the sequence of two myc-tags.

In an embodiment of the present invention, the inventors inserted an amino acid sequence that corresponds to a part of the myc protein (myc-tag) at several reasonable sites into the structure of a T cell receptor and transduced this modified receptor into T cells (see examples below). By introducing a tag into the TCR structure, it is possible to deplete the modified cells by administering the tag-specific antibody to the patient.

Those functional TCR fusion proteins may be used in a method for selecting a host cell population expressing a fusion protein selected from the group consisting of a fusion protein comprising a) at least one epitope-providing amino acid sequence (epitope-tag), and b) the amino acid sequence of an α- and/or β-chain of a TCR as defined above, wherein said epitope-tag is selected from an epitope-tag added to the N- and/or C-terminus of said α- and/or β-chain or added into the α- and/or β-chain sequence, but outside the CDR3 region, an epitope-tag inserted into a constant region of said α- and/or β-chain, and an epitope-tag replacing a number of amino acids in a constant region of said α- and/or β-chain; and a TCR comprising at least one fusion protein as above on the surface of the host cell; comprising contacting host cells in a sample with a binding agent that immunologically binds to the epitope-tag, and selection of said host cells based on said binding.

The present invention further provides an immunoglobulin molecule, anticaline, TCR γ/δ chain having a CDR3 region as defined herein (or a derivative thereof) inserted.

In a third aspect, the invention is directed to a T cell expressing a TCR as defined herein or a TCR comprising one of the CDR3 regions as defined above.

Furthermore, the invention provides a vector, preferably a plasmid, shuttle vector, phagemide, cosmid, expression vector, retroviral vector, adenoviral vector or particle and/or vector to be used in gene therapy, which comprises one or more of the nucleic acids as disclosed above.

In the context of the present invention, a "vector" shall mean a nucleic acid molecule as introduced into a host cell, thereby producing a transformed host cell. A vector may include nucleic acid sequences that permit it to replicate in a host cell, such as an origin of replication. A vector may also include one or more selectable marker genes and other genetic elements known to those of ordinary skill in the art. A vector preferably is an expression vector that includes a nucleic acid according to the present invention operably linked to sequences allowing for the expression of said nucleic acid.

A fourth aspect provides a cell, preferably a peripheral blood lymphocyte (PBL) which has been transformed with the above vector. The step of cloning the T cell receptor (TCR) of the

isolated T cells and/or expressing the TCR transgenes in PBMC can be done according to established methods such as those described in Engels et al., 2005.

In a fifth aspect, the present invention provides a pharmaceutical composition which comprises a TCR, a T cell or cell (PBL) as defined above and a pharmaceutically acceptable carrier.

Those active components of the present invention are preferably used in such a pharmaceutical composition, in doses mixed with an acceptable carrier or carrier material, that the disease can be treated or at least alleviated. Such a composition can (in addition to the active component and the carrier) include filling material, salts, buffer, stabilizers, solubilizers and other materials, which are known state of the art.

The term "pharmaceutically acceptable" defines a non-toxic material, which does not interfere with effectiveness of the biological activity of the active component. The choice of the carrier is dependent on the application.

The pharmaceutical composition can contain additional components which enhance the activity of the active component or which supplement the treatment. Such additional components and/or factors can be part of the pharmaceutical composition to achieve synergistic effects or to minimize adverse or unwanted effects.

Techniques for the formulation or preparation and application/medication of active components of the present invention are published in "Remington's Pharmaceutical Sciences", Mack Publishing Co., Easton, PA, latest edition. An appropriate application is a parenteral application, for example intramuscular, subcutaneous, intramedular injections as well as intrathecal, direct intraventricular, intravenous, intranodal, intraperitoneal or intratumoral injections. The intravenous injection is the preferred treatment of a patient.

According to a preferred embodiment, the pharmaceutical composition is an infusion or an injection.

An injectable composition is a pharmaceutically acceptable fluid composition comprising at least one active ingredient, e.g., an expanded T-cell population (for example autologous or

allogenic to the patient to be treated) expressing a TCR. The active ingredient is usually dissolved or suspended in a physiologically acceptable carrier, and the composition can additionally comprise minor amounts of one or more non-toxic auxiliary substances, such as emulsifying agents, preservatives, and pH buffering agents and the like. Such injectable compositions that are useful for use with the fusion proteins of this disclosure are conventional; appropriate formulations are well known to those of ordinary skill in the art.

In a further aspect, the present invention is directed to a method of treating a patient in need of adoptive cell therapy, said method comprising administering to said patient a pharmaceutical composition as defined above to said patient. The patient to be treated preferably belongs to the group of HLA-A2 positive patients.

Preferably, said patient suffers from a disease involving malignant cells expressing tyrosinase, preferably melanoma, glioma, glioblastoma, and/or rare tumors of ectodermal origin.

The present invention now will be illustrated by the enclosed Figures and the Examples. The following examples further illustrate the invention but, of course, should not be construed as limiting its scope.

DESCRIPTION OF THE FIGURES

Fig. 1: Screening of clones obtained from limiting dilution cultures after DC priming.
T cells were primed with dendritic cells expressing HLA-A2 and tyrosinase RNA. After two rounds of priming in vitro, cells were cloned by limiting dilution. 14 to 28 days later T cell clones showing adequate growth in individual culture wells were identified by light microscopy. Aliquots of growing clones were obtained and tested in a standard ^{51}Cr release assay to measure their killing activity against two melanoma target cell lines. Mel-A375 cells express HLA-A2 but not tyrosinase. Mel-93.04A12 cells express HLA-A2 and tyrosinase, so they can form the ligands recognized by HLA-A2-restricted, tyrosinase peptide-specific T cells. If Mel-A375 cells are recognized by T cell clones, this means the clones are alloreactive and recognize HLA-A2 independent of tyrosinase peptide (ie clone T41 and T42). If the T cell clones only recognize Mel-93.04A12, then they should have

specificity for HLA-A2-tyrosinase peptide ligands (i.e. T58, T43). Percentage specific lysis mediated by various T cell clones, (listed on x-axis) is given for the two target melanoma cell lines. The arrow designates clone T58 which shows strong killing of Mel-93.04A12 but not of Mel-A375. This clone was selected for further characterization based on its strong growth capacity.

Fig. 2: Comparison of clones T58 and IVS-B

Fig. 2a: Cytotoxic activity directed against melanoma cell lines.
The killing capacity of clone T58 was compared with that of clone IVS-B, derived from a melanoma patient, using as target cells the T2 cell line pulsed with synthetic tyrosinase-peptide for the amino acid sequence YMDGTMSQV in different molar concentrations, listed on the x-axis. The % relative lysis is given on the y-axis. The concentration of peptide that corresponds to 50% relative lysis is indicated by the crossing lines and shows that clone T58 can recognize substantially lower concentrations of peptide in comparison to clone IVS-B.

Fig. 2b: Measurement of multimer binding and off-rates.
The two clones were incubated with multimers to determine the percentage of positive cells at time 0 h. Both clones bound multimer on 100% of the cells. Multimer was washed out and the clones were incubated in medium containing HLA-A2-specific antibody. When multimers are released from the cell surface, they are captured by the antibody and can not rebind to the cells. The percent multimer-positive cells were reanalyzed at 1 h and 2 h.

Fig. 2c: Interferon-gamma secretion after stimulation with melanoma cell lines.
Clone T58 and IVS-B were co-cultured with the two melanoma cell lines used for the initial screening (described in Figure 1) and their secretion of IFN-γ into the culture medium was assessed by standard ELISA after 24 hours. n.d.= not detectable. Data are presented as pg/ml on the y-axis.

Fig. 2d: Cytotoxic activity against melanoma cell lines.
The clones were compared for killing activity using a standard ^{51}Cr-release assay as described in Figure 1. Data are given as percent specific lysis on the y-axis.

Fig. 3: Recognition of primary melanoma tumor cells by clone T58 and IVS-B.
(a) HLA-A2 surface expression on primary tumor cells (passage 12) of an HLA-A2$^-$ melanoma patient transfected with 50 µg HLA-A2 ivt-RNA and on established melanoma cell lines Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$) and Mel-A375 (HLA-A2$^+$tyrosinase$^-$) was measured by flow cytometry after staining with HLA-A2-specific monoclonal antibody. Each histogram shows the stained sample (filled curves) and the corresponding control sample (empty curves): control curves represent untransfected primary tumor cells stained with HLA-A2-specific monoclonal antibody (left histogram) or melanoma cell lines stained with isotype control antibody. HLA-A2 protein expression on RNA-transfected primary tumor cells was detected 10 h after electroporation. (b) The capacity of the patient-derived T cell clone (IVS-B), and T cell clone T58 to secrete IFN-γ or (c) release perforin in co-culture with the melanoma cells shown above was measured in ELISPOT assays.

Fig. 4: Transfer of antigen specificity by TCR retroviral gene transfer. (a) The human TCR-deficient T cell line Jurkat76[9] was transduced with the TCR of the T cell clone T58. TCR-expression was detected using tyrosinase-peptide-specific HLA-multimers. TCR expression was only detected in Jurkat76 cells tranduced with TCR-T58 (right histogram) and not in untransduced Jurkat76 cells (left histogram). (b) PBL of a healthy donor were retrovirally transduced with TCR-T58. After 10 days, untransduced and TCR-transduced PBL were analysed for tyrosinase TCR-expression using specific HLA-multimers. Multimer staining is shown on the x-axis and CD8 staining on the y-axis. The percentage of multimer$^+$CD8$^+$ T cells is displayed in the upper right quadrant. (c) Functionality of TCR-transduced PBL was measured using a standard IFN-γ release assay. T2 cells loaded with graded amounts of tyrosinase$_{369\text{-}377}$ peptide (10^{-12} M - 10^{-5} M) were used as target cells at a fixed effector to target cell ratio of 1:1. Untransduced PBL served as a control and showed no tyrosinase-peptide specific IFN-γ release (data not shown). Data are shown as pg/ml cytokine after subtration of secretion by untransduced PBL controls. (d) The capacity to secrete IFN-γ in co-culture with melanoma cell lines SK-Mel-28 (HLA-A2$^-$tyrosinase$^+$), Mel-A375 (HLA-A2$^+$tyrosinase$^-$), Mel-624.38 (HLA-A2$^-$tyrosinase$^+$) and Mel-93.04A12 (HLA-A2$^+$tyrosinase$^+$) was assessed using a standard IFN-γ release assay using an E:T = 1:1; (n.d. = not detectable).

Fig. 5: Transfer of specificity of T58 and IVS-B for HLA-A2 and tyrosinase-peptide YMDGTMSQV by TCR retroviral gene transfer. (a) PBL of a healthy donor were

retrovirally transduced with the patient-derived TCR-IVS-B or the TCR-T58. After 11 days, untransduced and TCR-transduced PBL were analysed for tyrosinase TCR-expression using specific HLA-multimers. Multimer staining is shown on the x-axis and CD8 staining on the y-axis. The percentage of multimer$^-$CD8$^+$ T cells is displayed in the upper right quadrant. (b) Functionality of TCR-transduced PBL was measured using a standard IFN-γ release assay. T2 cells loaded with graded amounts of tyrosinase$_{369-377}$ peptide (10^{-11} M - 10^{-5} M) or with 10^{-5} M irrelevant influenza matrix protein$_{58-66}$ were used as target cells at a fixed effector to target cell ratio of 1:1. Untransduced PBL served as a control and showed no tyrosinase-peptide specific IFN-γ release (data not shown). Data are shown as pg/ml cytokine after substration of secretion by untransduced PBL controls (mean = 318 pg/ml; range = 219-368 pg/ml) and adjustment for comparable numbers of multimer$^+$ cells.

Fig. 6: Tyrosinase peptide-specific CTL recognition of tumor cell lines and primary melanoma tumor cells. Columns represent the amount of IFN-γ (pg/ml) secreted by self-restricted D115 CTL and allo-restricted T58 CTL in co-culture with a panel of tumor cell lines from left to right: MaCa1 (HLA-A2-tyrosinase-); SK-Mel-28 (HLA-A2-tyrosinase+); Mel-A375, RCC-26, PancTu 1, MaCa1/A2, and UTS CC 1588 (all HLA-A2+tyrosinase-); Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29 and WM-266-4 (all HLA-A2+tyrosinase+). T cells designates CTL without stimulating cells. The HLA-A2+tyrosinase- tumor cell lines Mel-A375, RCC-26 and MaCa1/A2 were exogenously loaded with either 10-5 M irrelevant flu peptide or 10-5 M tyrosinase peptide YMD and IFN-γ secretion was measured by ELISA and given as pg/ml.

Fig. 7: Transfer of antigen specificity by retroviral transfer of TCR-D115 and TCR-T58. PBL of a healthy donor were transduced with TCR-D115 or TCR-T58. Specificity of recognition was assessed by IFN-γ release following co-culture with the tumor cell lines from left to right: MaCa1 (HLA-A2-tyrosinase-); SK-Mel-28 (HLA-A2-tyrosinase+); Mel-A375, RCC-26, PancTu 1, MaCa1/A2, and UTS CC 1588 (all HLA-A2+tyrosinase-); Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29 and WM-266-4 (all HLA-A2+tyrosinase+). T designates CTL without stimulating cells. The HLA-A2+tyrosinase- tumor cell lines Mel-A375, RCC-26 and MaCa1/A2 were exogenously loaded with either 10-5 M irrelevant flu peptide or 10-5 M tyrosinase peptide YMD and IFN-γ secretion was measured by ELISA and given as pg/ml.

Fig. 8: Transfer of antigen specificity by retroviral transfer of TCR-D115 and TCR-T58. (A) PBL of a healthy donor were transduced with TCR-D115 or TCR-T58. Unsorted TCR-transduced PBL were analyzed on day 10 for transgenic TCR-expression using irrelevant B7-pp65 and A2-pp65 multimers and specific A2-tyr multimers. Untransduced PBL showed no multimer binding (0.1%, data not shown). Percentages of multimer+CD8+ T cells are displayed in the upper right quadrant. (B) and (C) show the IFN-γ release of unsorted TCR-transduced PBL following stimulation with T2 cells loaded with graded amounts of tyrosinase peptide (10-12 M – 10-5 M) at a ratio of 2:1. In (B) the relative IFN-γ release is displayed in percent and in (C) the specific IFN-γ release is presented as pg/ml. (D) Functionality of unsorted TCR-transduced PBL was measured by IFN-γ release using autologous HLA-A2+ PBMC loaded with tyrosinase peptide (10-11 M – 10-6 M) as stimulating cells at ratio of 2:1. Untransduced PBL (▲) showed no peptide-specific IFN-γ release. (E) The HLA-A2+tyrosinase- tumor cell lines Mel-A375, RCC-26 and MaCa1/A2 were exogenously loaded with either 10-5 M irrelevant flu peptide (f) or 10-5 M tyrosinase peptide YMD (t) and IFN-γ secretion was measured by ELISA and given as pg/ml. (F) Specificity of recognition was assessed by IFN-γ release following co-culture with the tumor cell lines from left to right: MaCa1 (HLA-A2-tyrosinase-); SK-Mel-28 (HLA-A2-tyrosinase+); Mel-A375, RCC-26, PancTu 1, MaCa1/A2, and UTS CC 1588 (all HLA-A2+tyrosinase-); Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29 and WM-266-4 (all HLA-A2+tyrosinase+). T designates CTL without stimulating cells.

Fig. 9: TCR transfer retains differences in cytokine profile. (A-D) On the left hand side the cytokine release of TCR-transduced PBL in co-culture with the melanoma lines Mel-A375 (HLA-A2+tyrosinase-) and Mel-624.38 (HLA-A2+tyrosinase+) is depicted, on the right hand side the corresponding cytokine release after stimulation with T2 cells loaded with graded amounts of tyrosinase peptide (10-12 M – 10-5 M) is shown. Untransduced PBL (▲) showed no peptide-specific cytokine release. The following cytokines were measured: IFN-γ (A), IL-2 (B), TNF-α (C) and MIP-1β (D). The levels of cytokine secretion for all four cytokines were higher when PBL transduced with the allo-restricted TCR-T58 were used. Since untransduced PBL secreted very high levels of MIP-1β in response to T2 cells the peptide titration for this cytokine could not be evaluated.

EXAMPLE 1

The inventors prepared stimulating dendritic cells (DC) from an HLA-A2-negative healthy donor that co-expressed allogeneic HLA-A*0201-molecules and tyrosinase protein using mature DC that were electroporated with in vitro transcribed (ivt)-RNA for tyrosinase and HLA-A2, as described[1,2]. These DC were used to prime purified, autologous $CD8^+$ T cells using two rounds of stimulation with freshly prepared DC. After these two rounds of priming, $CD8^+$ T cells with T cell receptors (TCR) recognizing HLA-A2-tyrosinase$_{369-377}$-peptide complexes were stained using a tyrosinase$_{369-377}$/HLA-A*0201-multimer[3]. $CD8^+$multimer$^+$ cells were isolated by fluorescence activated cell sorting. Sorted cells were cloned in limiting dilution cultures and isolated clones showing HLA-A2/tyrosinase-peptide specificity were expanded using antigen-independent stimulation[4]. The T cell clone T58 was identified in an initial screen as having good functional activity (Figure 1).

Because T58 was isolated from an HLA-A*0201-negative donor it represents an allo-restricted T cell clone that did not undergo negative selection *in vivo*. The activity of the T58 clone was compared with the IVS-B clone that was isolated from a patient with metastatic melanoma[5]. This clone recognizes exactly the same HLA-A2/tyrosinase peptide ligand as clone T58 but it is self-restricted since it was activated *in vivo* in the patient who was HLA-A*0201-positive. This patient-derived T cell clone represents an example of T cells that are available in the peripheral repertoire that have undergone negative selection against self-peptides/self-MHC-molecules in the thymus *in vivo*.

Side-by-side comparisons of clone T58 and clone IVS-B were made to demonstrate the superior properties of the allo-restricted T58 clone versus the self-restricted IVS-B clone. Functional T cell avidity for tyrosinase$_{369-377}$ peptide recognition was measured in a ^{51}Cr-release assay using HLA-$A2^+$ T2 cells pulsed with graded amounts of exogenous peptide as target cells. The peptide concentration needed for 50% relative lysis defined the value of half-maximum lysis[6]. The allo-restricted T cell clone T58 required substantially less peptide to be activated by peptide-pulsed T2 cells than clone IVS-B ($6.0x10^{-10}$ M vs. $3.0x10^{-8}$ M) (Fig. 2*a*).

As an estimate of structural TCR-MHC/peptide binding affinity, loss of multimer binding was measured over time (*i.e.* HLA-multimer off-rate). A slower off-rate indicates that TCR-ligand interactions are more stable and of higher structural affinity[7]. After initial incubation with multimer and washing, T cells were incubated for 1 h and 2 h without multimers in the presence of HLA-A2-specific antibody to prevent cellular re-association of released multimers. The melanoma patient-derived T cell clone IVS-B showed an intermediate multimer binding: all cells were multimer$^+$ at 0 h and about 40% retained multimers at 1 and 2 h (Fig. 2*b*). In contrast, clone T58 had a slower off-rate, showing 74% positive binding at 1 h versus 41% for clone IVS-B and even at 2 h still had somewhat more multimer$^+$ cells (55% vs. 40%).

Both T cell clones were analyzed in an IFN-γ release assay for function and specificity (Fig. 2*c*). The clones were co-cultured with two melanoma cell lines that express HLA-A2 molecules but differ with respect to expression of tyrosinase protein: Mel-93.04A12 co-expresses both proteins (HLA-A2$^+$tyrosinase$^+$) but Mel-A375 fails to express tyrosinase protein (HLA-A2$^+$tyrosinase$^-$) and therefore can not generate the MHC-peptide ligand seen by the T cell clones. Allo-restricted T cell clone T58 was induced to secrete a high level of IFN-γ by the tyrosinase-expressing melanoma cell line, whereas only marginal cytokine secretion was seen with IVS-B cells (1,234 pg/ml vs. 106 pg/ml), demonstrating the vastly superior function of clone T58 in recognizing tumor cells expressing their HLA-A2-tyrosinase ligand. As expected, the clones showed no detectable IFN-γ secretion after stimulation with Mel-A375 cells, demonstrating the specificity for HLA-A2 and tyrosinase expression for tumor cell recognition.

The killing capacity of allo-restricted clone T58 was also compared with clone IVS-B using a ^{51}Cr-release assay (Fig. 2*d*). Again, clone T58 showed superior function (76% vs. 24% specific lysis).

Both clones were also tested for their capacity to recognize primary melanoma cells. Since primary HLA-A2$^+$ melanoma cells were not available, we introduced ivt-RNA for HLA-A2 into the tumor cells as for DC (Fig. 3*a*). Function was measured using ELISPOT assays detecting IFN-γ secretion and perforin release to bypass high spontaneous release of radioactive label by primary tumor cells. Recognition of primary tumor cells was shown to be HLA-A2-restricted since primary tumor cells lacking HLA-A2 RNA were not

recognized. Again, a strong difference was observed with poor recognition by the patient self-restricted IVS-B cells versus good recognition by allo-restricted T58 cells as assessed with IFN-γ secretion (Fig. 3*b*) and by perforin secretion (Fig. 3*c*).

To demonstrate that the superior functional avidity of allo-restricted T58 cells resided directly in the TCR, separate recombinant retroviruses were created for TCR alpha and beta chains of clone T58 as described[8]. Human TCR-deficient Jurkat76 cells[9] were co-infected with the α-chain and β-chain retroviruses and transgenic TCR-expression was measured by multimer staining. TCR-T58 was expressed at a good level, demonstrating adequate quality of the separate retroviral supernatants (Fig. 4*a*). Next, activated peripheral blood lymphocytes (PBL) of a healthy HLA-A2$^-$ donor were transduced and analyzed with multimers for tyrosinase-specific TCR-expression (Fig. 4*b*). Despite this low frequency, PBL transduced with TCR-T58 released high amounts of IFN-γ following stimulation with T2 cells pulsed with graded amounts of tyrosinase-peptide (Fig. 4*c*). TCR-T58 transduced PBL could also respond specifically to stimulation by melanoma cell lines that expressed HLA-A2 and tyrosinase (Fig. 4*d*). They did not respond to tumor cells that did not express HLA-A2 or tyrosinase, again demonstrating the specificity of HLA-A2-tyrosinase ligands for T58 recognition.

Bi-cistronic retroviral vectors were also prepared encoding the α-chain and β-chains of the TCR of IVS-B cells and used to transduce activated PBL. In parallel, the same activated PBL were transduced with bi-cistronic retroviral vectors encoding the two chains of TCR-T58. PBL expressing the corresponding receptors were identified by co-staining for CD8 and multimer and showed low numbers of positive cells. (Fig. 5*a*) Despite their low frequency, PBL transduced with TCR-T58 released high amounts of IFN-γ following stimulation with T2 cells pulsed with graded amounts of tyrosinase-peptide. PBL expressing TCR-IVS-B secreted far less IFN-γ. Tyrosinase peptide-specific cytokine secretion was not detected with untransduced PBL control cells. Data are shown as pg/ml cytokine after substraction of secretion by untransduced PBL controls (mean = 318; range = 219 – 369 pg/ml) (Fig. 5*b*).

Table 1 shows the genetic information regarding the use of VJ and VDJ gene segments by the alpha and beta chains of TCR-T58, respectively. The CDR3 regions, according to

IMGT, are presented as nucleotide sequences and amino acid sequences. Also shown are the codon optimized sequences for the full VJ and VDJ regions.

Materials and Methods

Cell lines

The human melanoma cell lines, Mel-A375 (HLA-A2$^+$, tyrosinase$^-$; CRL-1619, American Type Culture Collection (ATCC), Bethesda, MD), Mel-93.04A12 (HLA-A2$^+$, tyrosinase$^+$, gift of P. Schrier, Department of Immunohematology, Leiden University Hospital, The Netherlands), Mel-624.38[10] (HLA-A2$^+$, tyrosinase$^+$, gift of M. C. Panelli, National Institutes of Health, Bethesda, MD), SK-Mel-28 (HLA-A2$^-$, tyrosinase$^+$; MTB-72, ATCC) as well as the lymphoid cell line T2 (CRL-1992, ATCC), and the human TCR-deficient Jurkat76[9] T cell line were cultured in RPMI 1640 medium supplemented with 12% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium-pyruvate and non-essential amino acids.

The HLA-A*0201-restricted tyrosinase$_{369-377}$ peptide-specific melanoma patient-derived IVS-B T cell clone was cultured as described[5].

Production of tyrosinase and HLA-A2 *ivt*-RNA

The plasmid pCDM8-HLA-A2 with HLA-A*0201 cDNA and the pZeoSV2+/huTyr with tyrosinase cDNA were linearized and used as *in vitro* transcription templates to produce RNA with the aid of the mMESSAGE mMACHINE T7 kit (Ambion, Austin, TX) according to the manufacturer's instructions.

***De novo* priming of T cells with RNA-pulsed DC**

Blood samples from healthy donors were collected after informed consent and with approval of the Institutional Review Board of the University Hospital of the Ludwig-Maximilians-University, Munich, Germany. Peripheral blood lymphocytes (PBL) were isolated by Ficoll density gradient centrifugation. PBL were resuspended in 15 ml very low

endotoxin (VLE) RPMI 1640 medium (Biochrom, Berlin, Germany) supplemented with 1.5% human serum (DC medium) at 7.5×10^7 cells per 75 cm^2 culture flask and incubated at 37°C and 5% CO_2 for 1 h. Non-adherent cells were carefully removed by washing. Mature DC were prepared from adherent monocytes and transfected with ivt-RNA *via* electroporation as previously described[2]. DC of HLA-$A2^-$ donors were co-transfected with 24 µg tyrosinase ivt-RNA and 48 µg HLA-A2 ivt-RNA. On the same day, autologous $CD8^+$ T lymphocytes were enriched from PBL *via* negative selection using a commercial kit according to the manufacturer's instructions ($CD8^+$ T cell Isolation Kit II (human), Miltenyi, Bergisch Gladbach, Germany). Co-cultures were initiated 10 h after DC electroporation in 24-well plates (TPP, Trasadingen, Switzerland) by adding $1x10^5$ RNA-pulsed DC to $1x10^6$ $CD8^+$ T cells in RPMI 1640, supplemented with 10% heat-inactivated human serum, 4 mM L-glutamine, 12.5 mM HEPES, 50 µM β-mercaptoethanol and 100 U/ml penicillin/streptomycin (T cell medium). IL-7 (5 ng/ml) (Promokine, Heidelberg, Germany) was added on day 0 and 50 U/ml IL-2 (Chiron Behring, Marburg, Germany) was added after 2 days and then on every 3rd subsequent day. Addition of IL-2 was delayed to decrease proliferation of non-specific $CD8^+$ T cells[4]. The 2nd stimulation of primed T cells was made after seven days using freshly prepared RNA-pulsed DC.

HLA-multimer staining and sorting

Seven days after the 2nd stimulation of CD8-enriched T cells with RNA-pulsed DC, HLA-A2-restricted tyrosinase-specific T cells were detected by staining with a PE-labeled HLA-A*0201/$htyr_{369-377}$ peptide/human β_2m multimer[11], anti-CD8-APC antibody (clone RPA-T8, BD Pharmingen, Franklin Lakes, NJ) and propidium iodide (PI: 2 µg/ml). For sorting, up to $5x10^6$ cells were incubated with 12 µg multimer in 100 µl PBS + 0.5% human serum. CD8-APC antibody was then added at 1/50 for an additional 25 min. After staining cells were washed twice and diluted in PBS + 0.5% human serum with PI for sorting. 20-$50x10^6$ total cells per priming culture were stained for sorting. PI-negative cells were gated and $CD8^+$multimer$^+$ T cells were sorted on a FACSAria cell sorter (BD Biosciences) with a 70 µm nozzle, at a rate of 15,000 events/s.

For HLA-multimer off-rate assays, cells were washed after multimer binding and resuspended in FACS buffer containing saturating amounts of BB7.2 monoclonal antibody (ATCC) to capture detached multimers and prevent rebinding to T cells. After 1 or 2 h,

samples were fixed in FACS buffer with 1% paraformaldehyde and analysed by flow cytometry[7].

Culture of peptide-specific T clones

Multimer-sorted T cells were cloned by limiting dilution. Clones were plated in 96-well round-bottom plates (TPP) in 200 µl/well T cell medium. 50 IU/ml IL-2 was supplemented every 3 days with 5 ng/ml IL-7 and 10 ng/ml IL-15 (PeproTech Inc., Rocky Hill, NJ) every 7 days. T cell clones were stimulated non-specifically with anti-CD3 antibody (0.1 µg/ml; OKT-3) and provided with $1x10^5$ feeder cells per 96-well, consisting of irradiated (50 Gy) PBL derived from a pool of five unrelated donors and $1x10^4$ irradiated (150 Gy) EBV-transformed allogeneic B-LCL every two weeks. Proliferating T cells were transferred into 24-well plates (TPP) and cultured in 1.5 ml T cell medium plus cytokines. $1x10^6$ allogeneic irradiated PBL and $1x10^5$ irradiated EBV-transformed allogeneic B-LCL were added per well as feeder cells in 24-well plates. Clonality was determined by TCR-beta-chain receptor analysis, as described[12].

Peptide loading of T2 cells

For exogenous peptide pulsing, $1x10^6$ T2 cells were incubated at 37°C and 5% CO_2 for 2 h with 10 µg/ml human β_2-microglobulin (Calbiochem, San Diego, CA) and titrating amounts, ranging from 10^{-5} M to 10^{-12} M, of the tyrosinase peptide YMD (tyrosinase$_{369-377}$ YMDGTMSQV, Metabion, Martinsried, Germany). T2 cells pulsed with 10^{-5} M influenza peptide GIL (influenza matrix protein$_{58-66}$ GILGFVTL, Metabion) served as negative control. After washing, peptide-loaded T2 cells were used as target cells in cytotoxicity or IFN-γ-release assays.

IFN-γ release assay

For investigation of specificity, T cell clones (2×10^3 cells in 100 µl) were incubated with the respective melanoma cell lines or peptide-pulsed T2 cells (1×10^4 cells in 100 µl). Culture supernatants were harvested after 24 h co-culture and assessed by a standard ELISA using the OptEIA™ Human IFN-γ Set (BD Biosciences Pharmingen).

Cytotoxicity assay

Cytotoxic activity of T cell clones was analysed in a standard 4 h 51-chromium release assay. Melanoma cells or peptide-loaded T2 cells were used as target cells. Briefly, $1x10^6$ target cells were labeled with 100 µCi $Na_2{}^{51}CrO_4$ (ICN Biochemicals, Irvine, CA) for 1-1.5 h. ^{51}Cr-labeled target cells were cultured with T cells in 100 µl/well RPMI 1640 with 12% FCS in V-bottom 96-well tissue culture plates (Greiner, Solingen, Germany). For determination of functional avidity $1x10^4$ T cells were added to $1x10^3$ peptide-pulsed T2 cells loaded with titrated amounts of peptide, giving a constant E:T of 10:1.

After 4 h co-culture at 37°C, 50 µl of supernatant were collected and radioactivity was measured in a gamma counter. The percentage of specific lysis was calculated as: 100 x (experimental release – spontaneous release)/ (maximum release – spontaneous release). Spontaneous release was assessed by incubating target cells in the absence of effector cells and was generally less than 15%. For the calculation of percent relative lysis, the maximum percent specific lysis was set to the reference value of 100% and corresponding values were calculated corresponding to this reference. To determine half-maximum lysis, percent relative lysis was plotted against peptide concentration. The peptide concentration at which the curve crossed 50% relative lysis was taken as the value of half-maximum lysis[6].

ELISPOT

Antibody pre-coated PVDF plates (Mabtech AB, Nacka, Sweden) were incubated at 37°C in CTL Test™ medium (Cellular Technology Ltd., Cleveland, Ohio) for 2 h to block unspecific binding. For the IFN-γ ELISPOT, plates were pre-coated with the IFN-γ-specific capture antibody clone 1-D1K; for perforin ELISPOT plates were pre-coated with the perforin-specific capture antibody (clone Pf-80/164; Mabtech AB). Primed T cells were washed with CTL Wash™ Supplement culture medium (Cellular Technology Ltd) and $1x10^3$ responder T cells were stimulated with $5x10^3$ melanoma cells in 150 µl CTL Test™ medium and 24 h later assessed in IFN-γ ELISPOT or 48 h later in perforin ELISPOT. After washing with PBS/0.01% Tween and PBS alone, plates were incubated either with a direct streptavidin-alkaline phosphatase (ALP)-conjugated detection antibody (clone 7-B6-1; Mabtech AB) for IFN-γ ELISPOT or with biotinylated detection antibody (clone Pf-344;

Mabtech AB) for perforin ELISPOT for 2 h at room temperature following a 1 h incubation with streptavidin-alkaline phosphatase (ALP). The plates were washed again and a ready-to-use BCIP/NBT-plus substrate solution (Mabtech AB) was added. Spots were counted using the AID reader system ELR03 with the software version 4.0 (AID Autoimmun Diagnostika GmbH, Strassberg, Germany).

Construction of retroviral vectors, production of virus supernatants and transduction of Jurkat76 T cells and PBL

For TCR identification of tumor-specific T cell clones, part of the TCRα- and TCRβ-chain sequences including the complementary determining region (CDR3) was amplified by PCR using a panel of TCRVα and TCRVβ primers combined with the respective constant region primer as described[13]. The TCRα and TCRβ chain genes of T cell clones T58 and IVS-B were amplified by PCR with gene specific primers and cloned into the retroviral vector MP71PRE[8] via NotI and EcoRI restriction sites. Both chains of human TCR-T58 (Vα7, Vβ23) and TCR-IVS-B (Vα3, Vβ14) were constructed as single-TCR gene vectors or double-TCR gene vectors (pMP71-T58α and pMP71-T58β, pMP71-IVS-Bα and pMP71-IVS-Bβ; pMP71-T58β-P2A-T58α and pMP71-IVS-Bβ-P2A-IVS-Bα). Retroviral vector plasmids were co-transfected into 293T cells with expression plasmids encoding Moloney MLV gag/pol and MLV-10A1 env gene to produce amphotropic MLV-pseudotyped retroviruses as described[14]. The human TCR-deficient T cell line Jurkat76 and PBL were transduced as reported[14]. Jurkat76 cells (5 days after transduction) and PBL (10 days after transduction) were stained using PE-labeled HLA-A*0201/htyr$_{369-377}$ peptide/human β_2m multimer and anti-CD8-FITC antibody. On day 13 an IFN-γ release assay was performed using T2 cells loaded with graded amounts of tyrosinase$_{369-377}$ peptide (10^{-12} M - 10^{-5} M) or T2 cells pulsed with 10^{-5} M influenza matrix protein$_{58-66}$ peptide and the tumor cell lines SK-Mel-28, Mel-A375, Mel-624.38 and Mel-93.04A12 as stimulating cells at an E:T ratio = 1:1. Control values for peptide-stimulated untransduced PBL were subtracted from values of transduced cells at each peptide concentration and then adjusted to comparable numbers of total TCR-transgenic cells.

T58-TCR analysis

For the T-cell receptor analysis of the tyrosinase-specific clone T58, part of the TCR alpha-chain and beta-chain containing the CDR3 region was amplified by RT-PCR using a panel of TCR Vα and TCR Vβ primers combined with a respective TCR constant region primer. Products were sequenced and assigned according to IMGT (Table 1; IMGT, the international ImMunoGeneTics information system®, http://imgt.cines.fr).

Modifications of the TCR-sequence

Codon optimization of the VJ/VDJ-regions of both T58-TCR chains was done to facilitate TCR mRNA translation (Table 1). Antibody-tags, for example myc-tags[15] (Patent Application number: 06014606.5-1212) or other modifications, for example a CD20 epitope, can be introduced in any position, i.e. the N-terminus of the TCRα-chain, that is recognized by the depleting antibody and does not interfere with TCR-functionality.

Table 1. TCR-CDR3 sequences and codon optimized VJ/VDJ regions of clone T58

	Alpha-chain
VJ region*	TRAV1-2 AJ28
CDR3 region* Nucleotide sequence Amino acid sequence	 TGTGCTGTGACATACTCTGGGGCTGGGAGTTACCAACTC (SEQ ID NO: 3) C A V T Y S G A G S Y Q L (SEQ ID NO: 5)
Codon optimized VJ	ATGTGGGGCGTGTTTCTGCTGTACGTGTCCATGAAGATGGGCGGCACCACCGGCCAGAACATCGACCAGCCCACCGAGATGACAGCCACCGAGGGCGCCATCGTGCAGATCAACTGCACCTACCAGACCAGCGGCTTCAACGGCCTGTTCTGGTATCAGCAGCACGCCGGCGAGGCCCCTACCTTCCTGAGCTACAACGTGCTGGACGGCCTGGAAGAGAAGGGCCGGTTCAGCAGCTTCCTGAGCCGGTCCAAGGGCTACAGCTACCTGCTGCTGAAAGAACTGCAGATGAAGGACAGCGCCAGCTACCTGTGCGCCGTGACCTACAGCGGAGCCGGCAGCTACCAGCTGACCTTCGGCAAGGGCACCAAGCTGTCCGTG (SEQ ID NO: 7)

	Beta-chain
VDJ region*	TRBV13 BD1 BJ1-4
CDR3 region* Nucleotide sequence Amino acid sequence	 TGTGCCAGCAGTCAGAAACAGGGCTGGGAAAAACTG (SEQ ID NO: 4) C A S S Q K Q G W E K L (SEQ ID NO: 6)
Codon optimized VDJ	ATGCTGTCCCCCGATCTGCCCGACAGCGCCTGGAACAC CAGACTGCTGTGCCACGTGATGCTGTGTCTGCTGGGAG CCGGATCTGTGGCCGCTGGCGTGATCCAGAGCCCCAG ACACCTGATCAAAGAGAAGCGGGAGACAGCCACCCTG AAGTGCTACCCCATCCCCCGGCACGACACCGTGTACTG GTATCAGCAGGGACCAGGACAGGACCCCCAGTTCCTG ATCAGCTTCTACGAGAAGATGCAGAGCGACAAGGGCA GCATCCCCGACAGATTCAGCGCCCAGCAGTTCAGCGA CTACCACAGCGAGCTGAACATGAGCAGCCTGGAACTG GGCGACTCTGCCCTGTACTTCTGCGCCAGCAGCCAGAA GCAGGGCTGGGAGAAGCTGTTCTTCGGCAGCGGCACC CAGCTGTCCGTGCTG (SEQ ID NO: 8)

TCR alpha-chain (VJ region), TCR beta-chain (VDJ region) and CDR3 lenghts are designated according to IMGT (IMGT, the international ImMunoGeneTics information system ®, http://imgt.cines.fr)

Example 2

In Example 1, data are provided that compared two T cell clones that specifically recognize a peptide derived from tyrosinase (ie YMDGTMSQV hereafter referred to as YMD) presented by HLA-A*0201 molecules. The T cell clone T58 was an allo-restricted, peptide-specific T cell clone derived from an HLA-A2-negative donor. The T cell clone IVS-B was derived from an HLA-A*0201-positive patient who suffered from metastatic melanoma. This melanoma expressed tyrosinase.

In this Example, comparisons have been extended to include an example of a T cell clone, D115, which is also derived from an HLA-A*0201-positive individual and recognizes the same YMD peptide. However, in contrast to clone IVS-B, clone D115 was generated in vitro using responding T cells derived from the blood of a healthy individual. Therefore, there have been no potential negative impacts on this T cell clone from a tumor environment (ie melanoma) in vivo.

Figure 6 shows a comparison of the pattern of the target cell recognition of the new clone D115 and clone T58 which is the subject of this patent. As can be clearly seen, both D115 and T58 show the same pattern of recognition, detected by secretion of interferon-gamma (y-axis), after co-cultivation with various tumor cell lines (x-axis and figure legend). Neither clone recognizes tumor cells that are HLA-A2-negative but express tyrosinase, nor do they recognize tumor cells that are HLA-A2-positive and tyrosinase negative. On the other hand, both T cell clones recognize several tumor cell lines that are both HLA-A2-positive and tyrosinase-positive. The role of the YMD peptide in this recognition is shown by the finding that HLA-A2-positive tumor cells that do not express tyrosinase from which the YMD peptide could be processed internally and transported to the cell surface by HLA-A2 molecules for presentation, can be loaded with synthetic YMD peptide, leading to their recognition by D115 and T58. Thereby, both clones show the same specificity for the YMD peptide presented by HLA-A2 molecules. However, the efficiency of recognition displayed by clone T58 is far superior to clone D115, as seen by the levels of interferon-gamma secretion. This, for example, leads to negligible recognition of the melanoma cell line SK-Mel-29 by D115 but clear recognition by T58.

The TCR of clone D115 and T58 were expressed as recombinant proteins in activated recipient lymphocytes (Figure 7). When these TCR-transduced lymphocytes were retested with the same panel of target cells, they showed the same specificity pattern as the original T cell clones, demonstrating that the TCR recognition was responsible for the results seen in Figure 6. Again, in Figure 7 it is demonstrated that the TCR of clone T58 shows superior recognition of the melanoma tumor cell lines that express HLA-A2 and tyrosinase and the YMD peptide-pulsed HLA-A2-positive tumor cells.

Figure 8A shows that the TCR-transduced lymphocytes show comparable levels of expression of the respective recombinant TCRs, with each transduced population having

around 11% of T cells that bind a MHC multimer comprised of HLA-A2 molecules presenting the YMD peptide. Such binding is not observed with control multimers that present other peptides derived from the pp65 protein of human cytomegalovirus.

When the two populations of TCR-transduced PBL are stimulated with HLA-A2-positive antigen-presenting cells (ie T2 cells) that are pulsed with different concentrations of YMD peptide (shown on the x-axis), it can be seen that the cells expressing TCR-T58 release 50% of their maximal levels of interferon-gamma (y-axis) at 100-fold lower peptide concentrations. This peptide-sensitivity assay shows that the TCR-T58 has a much higher functional avidity when compared to TCR-D115 (Figure 8B).

This difference is further exemplified by the strong difference in the maximum levels of interferon-gamma produced by the TCR-T58- versus TCR-D115-transduced lymphocytes. In the case of TCR-T58 cells, the maximum reaches 5000 pg/ml whereas this results in only around 2000 pg/ml for TCR-D115 in 24 hours. Furthermore, the amount of peptide that must be presented by T2 cells to cause release of 2000 pg/ml interferon-gamma is 15,000-fold lower for triggering of this level of response from TCR-T58-transduced lymphocytes compared with TCR-D115-transduced lymphocytes (Figure 8C).

Figure 8D shows another peptide-sensitivity assay, this time using peripheral blood mononuclear cells that have been pulsed with titrating amounts of YMD peptide (x-axis). Once again, the amounts of interferon-gamma released by lymphocytes expressing TCR-T58 are much greater compared with TCR-D115. The arrows show that the first detection of cytokine secretion occurs with 1000-fold less peptide for TCR-T58 compared with TCR-D115.

Figures 8E and 8F demonstrate the specificity of the transduced lymphocyte populations for peptide-pulsed tumor cells (Figure 8E) or tumor cell lines expressing HLA-A2 and tyrosinase (Figure 8F). In all cases, recognition is superior by lymphocytes expressing TCR-T58 compared to TCR-D115.

The superior secretion of cytokine is not limited to interferon-gamma. The levels of secretion of interleukin-2, TNF-alpha and MIP-1beta are also superior for TCR-T58. This is

seen after stimulation of the TCR-transduced lymphocytes by tumor cells or by peptide-pulsed T2 cells (Figure 9).

Material and methods

Cell lines

The human melanoma cell lines, Mel-A375 (HLA-A2$^+$, tyrosinase$^-$; CRL-1619, American Type Culture Collection (ATCC)), Mel-93.04A12 (HLA-A2$^+$, tyrosinase$^-$; gift of P. Schrier, Department of Immunohematology, Leiden University Hospital, The Netherlands), Mel-624.38[1] and SK-Mel-23 (HLA-A2$^+$, tyrosinase$^-$; gift of M. C. Panelli, National Institutes of Health, Bethesda, MD), SK-Mel-28 (HLA-A2$^-$, tyrosinase$^+$; MTB-72, ATCC), SK-Mel-29 (HLA-A2$^+$, tyrosinase$^+$, gift of P. Rieber, Institute of Immunology, Technical University Dresden, Germany), WM-266-4 (HLA-A2$^+$, tyrosinase$^+$; CRL-1676, ATCC) and primary cultures of a human melanoma (passage 6-12) and MaCa1 (HLA-A2$^-$, tyrosinase$^-$, gift of R. Wank, M.D. Munich, Germany), stable HLA-A*0201 transfectant of MaCa1 (MaCa1/A2) (HLA-A2$^+$, tyrosinase$^-$, gift of E. Noessner, Institute of Molecular Immunology, Helmholtz Zentrum München, Germany), RCC-26[2] (HLA-A2$^+$, tyrosinase$^-$), PancTu1 (HLA-A2$^+$, tyrosinase$^-$, gift of P. Nelson, Department for Biological Chemistry University Hospital LMU Munich, Germany), UTS CC 1588 (HLA-A2$^+$, tyrosinase$^-$, gift of M. Schmitz, Institute of Immunology, Technical University Dresden, Germany) as well as the lymphoid cell line T2 (CRL-1992, ATCC) were cultured in RPMI 1640 medium supplemented with 12% fetal bovine serum (FBS), 2 mM L-glutamine and 1 mM sodium-pyruvate and non-essential amino acids.

Peptide loading of T2 cells, PBMC and tumor cells

For exogenous peptide pulsing, $1x10^6$ T2 cells were incubated at 37°C and 5% CO_2 for 2 h with 10 µg/ml human β_2-microglobulin (Calbiochem) and titrating amounts, ranging from 10^{-5} M to 10^{-11} M, of the tyrosinase peptide YMD ($tyrosinase_{369-377}$ YMDGTMSQV, Metabion). T2 cells pulsed with 10^{-5} M influenza peptide GIL (flu: influenza matrix $protein_{58-66}$ GILGFVFTL, Metabion) served as the negative control. PBMC were loaded with tyrosinase peptide as for T2 cells with titrating amounts ranging from 10^{-6} to 10^{-11} M. Tumor cells were loaded with either 10^{-5} M flu peptide or 10^{-5} M tyrosinase peptide YMD

as described for T2 cells. After washing, peptide-loaded T2 cells, PBMC or tumor cells were used as stimulating cells in IFN-γ release assays.

Cytokine assays

For investigation of specificity, CTL (2×10^3 cells in 100 µl) were incubated with various tumor cell lines (1×10^4 cells in 100 µl), with or without peptide pulsing, as described above. Culture supernatants were harvested after 24 h co-culture and assessed by a standard ELISA using the OptEIA™ Human IFN-γ Set (BD Biosciences). Data represent mean values with corresponding mean deviations calculated from duplicate determinations. For the calculation of % relative IFN-γ release, the maximum IFN-γ release was set to the reference value of 100% and corresponding values were calculated corresponding to this reference.
To investigate multiple cytokines simultaneously (IFN-γ, IL-2, TNF-α and MIP-1β) cytokine secretion in supernatants of co-culture of CTL with tumor cells and with or without tyrosinase peptide pulsed T2 cells (10^{-5} M) was measured using the multiplex protein array system technology (Bio-Rad Laboratories, Hercules, CA).

Retroviral TCR gene transfer

For TCR identification of tumor-specific CTL, regions of the TCRα- and TCRβ-chains encoding CDR3 were amplified by PCR using a panel of TCRVα and TCRVβ primers in combination with respective constant region primers as described.[3] The full TCRα- and TCRβ-chain genes of CTL clones T58 and D115 were amplified by PCR using cDNA as template. Primer sequences will be provided on request. The constant regions of both TCR chains were exchanged by the murine counterparts to increase the stability of the TCR.[4] The TCR chains were linked by a 2A peptide linker (TCRβ-P2A-TCRα)[5], codon-optimized (Geneart)[6] and cloned into the retroviral vector MP71PRE via *Not*I and *Eco*RI restriction sites.[5] Retroviral vector plasmids were co-transfected into 293T cells with expression plasmids encoding Moloney MLV gag/pol and MLV-10A1 env gene, respectively, to produce amphotropic MLV-pseudotyped retroviruses as described.[5] Ten days after the second transduction, PBL were stained using PE-labeled A2-tyr multimer and FITC-labeled CD8-specific antibody. Multimers presenting peptides derived from cytomegalovirus pp65 were used as controls: PE-labeled HLA-B7 $pp65_{417-427}$ (B7-pp65) multimers served as the HLA control and HLA-A2 $pp65_{495-503}$ multimers as a peptide-specificity control. On day 15

an IFN-γ release assay was performed using T2 cells or autologous PBMC loaded with graded amounts of tyrosinase peptide (10^{-12} M – 10^{-5} M) and the tumor cell lines MaCa1, SK-Mel-28, Mel-A375, RCC-26, PancTu 1, MaCa1/A2, UTS CC 1588, Mel-624.38, Mel-93.04A12, SK-Mel-23, SK-Mel-29 and WM-266-4 as stimulating cells at an E:T of 2:1.

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Patent Claims:

1. A nucleic acid molecule coding for the V(D)J regions of a TCR that recognizes tyrosinase and comprising the nucleic acid sequence of SEQ ID NO: 1 coding for the α-chain and/or the nucleic acid sequence of SEQ ID NO: 2 coding for the β-chain of said TCR, or a derivative thereof, coding for the α- or β-chain, wherein the chain has been altered by one or more additions or deletions of from 1-15 amino acids, the additions or deletions being outside the CDR3 region of each chain and/or by conservative substitutions of from 1-15 amino acids, wherein tyrosinase recognizing characteristics are maintained or improved,
or
a fragment thereof coding for a CDR3 region of a TCR recognizing tyrosinase and having the nucleic acid sequence of SEQ ID NO: 3 or 4 or coding for the amino acid sequences of SEQ ID NO: 5 or 6,
or
a derivative of said fragment, wherein the CDR3 region has been altered by one or more additions and/or deletions of an overall number of from 1-5 amino acids, but not more than 1-3 contiguous amino acids and/or conservative substitutions of from 1-6 amino acids and wherein the tyrosinase recognizing characteristics are maintained or improved.

2. The isolated nucleic acid molecule of claim 1, wherein the derivative of the α- or β-chain coding sequence is derived from SEQ ID NO: 1 and 2 by codon optimization and comprises the nucleic acid sequence of SEQ ID NO: 7 coding for the α-chain and/or comprises the nucleic acid sequence of SEQ ID NO: 8 coding for the β-chain of said TCR.

3. A TCR, preferably a soluble TCR, encoded by a nucleic acid of one or more of claims 1-2 or comprising the amino acid sequences of SEQ ID NO: 5 and/or 6.

4. A functional TCR α and/or β chain fusion protein, comprising:
a) at least one epitope-tag, and
b) the amino acid sequence of an α and/or β chain of a TCR according to one or more of claims 1-3,
wherein said epitope-tag is selected from

i) an epitope-tag added to the N- and/or C-terminus of said α and/or β chain, or added into the α and/or β chain sequence, but outside the CDR3 region,
ii) an epitope-tag inserted into a constant region of said α and/or β chain, and
iii) an epitope-tag replacing a number of amino acids in a constant region of said α and/or β chain.

5. A T cell expressing a TCR of claim 3 or 4, or a TCR comprising one of the CDR3 regions of claim 1.

6. An immunoglobulin molecule, anticaline, TCR γ/δ chain having a CDR3 region of claim 1 inserted.

7. A vector, preferably a plasmid, shuttle vector, phagemide, cosmid, expression vector, retroviral vector, adenoviral vector or particle and/or vector to be used in gene therapy, which comprises one or more of the nucleic acids of one of claims 1 - 2 or a nucleic acid coding for a TCR according to claim 3 or 4.

8. A cell, preferably a PBL which has been transformed with the vector of claim 7.

9. A pharmaceutical composition which comprises a TCR of claim 3 or 4, a T cell of claim 5, an immunoglobulin molecule, anticaline, TCR γ/δ chain of claim 6 or the cell of claim 8 and a pharmaceutically acceptable carrier, preferably an infusion or injection.

10. The use of pharmaceutical composition in accordance with claim 9 for the manufacture of a medicament for use in adoptive cell therapy.

11. The use of claim 10, wherein the medicament is for treating a disease in patients, preferably HLA-A2 positive patients, the disease involving malignant cells expressing tyrosinase, preferably melanomas, gliomas, glioblastomas, and/or rare tumors of ectodermal origin.