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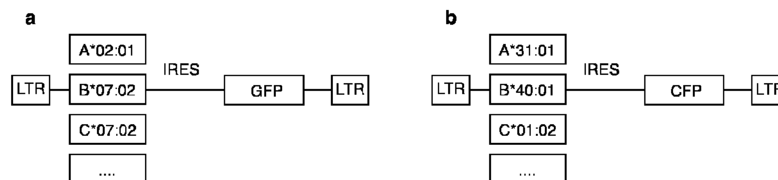
(54) **Title**: METHOD OF DETECTING NEW IMMUNOGENIC T CELL EPITOPES AND ISOLATING NEW ANTIGEN-SPECIFIC T CELL RECEPTORS BY MEANS OF AN MHC CELL LIBRARY

Fig. 1



(57) **Abstract**: The present invention relates to the field of immunotherapy, in particular, to adoptive T cell therapy, T cell receptor (TCR) gene therapyand vaccination. The invention provides a method for preparing a nucleic acid encoding the TCR alpha chain construct (TRA) and TCR beta chain construct (TRB) of a TCR construct specific for an epitope from anantigen presented on major histocompatibility complex (MHC), comprising contacting T cells isolated from a donor with a library of artificial antigen presenting cells (APC)comprising cells expressing all MHC I or MHC II alleles present in the donor, preferably, in K562 cells. The TCR construct can be expressed in a T cell,which is useful for adoptive T cell therapy, e.g., of cancer,viral infections or autoimmune diseases. The invention further provides a method for identifying the epitope recognized by said TCR. Immunogenic epitopes recognized by said TCRs can be used to develop vaccine formulations to induce antigen-specific T cell immunity in patients. The invention further provides pairs of two TCRconstructs and respective immunogenic epitopes obtained by the method of the invention, wherein the epitopesare from human papillomavirus (HPV) 16 (also designated alphapapillomavirus 9) oncoprotein E5 and human cytomegalovirus (CMV) protein pp65.

Method of detecting new immunogenic T cell epitopes and isolating new antigen-specific T cell receptors by means of an MHC cell library

The present invention relates to the field of immunotherapy, in particular, to adoptive T cell therapy, T cell receptor (TCR) gene therapy and vaccination. The invention provides a method for preparing a nucleic acid encoding the TCR alpha chain construct (TRA) and TCR beta chain construct (TRB) of a TCR construct specific for an epitope from an antigen presented on major histocompatibility complex (MHC), comprising contacting T cells isolated from a donor with a library of artificial antigen presenting cells (APC) comprising cells expressing all MHC I or MHC II alleles present in the donor, preferably, in K562 cells. The TCR construct can be expressed in a T cell, which is useful for adoptive T cell therapy, e.g., of cancer, viral infections or autoimmune diseases. The invention further provides a method for identifying the epitope recognized by said TCR. Immunogenic epitopes recognized by said TCRs can be used to develop vaccine formulations to induce antigen-specific T cell immunity in patients. The invention further provides pairs of two TCR constructs and respective immunogenic epitopes obtained by the method of the invention, wherein the epitopes are from human papillomavirus (HPV) 16 (also designated alphapapillomavirus 9) oncoprotein E5 and human cytomegalovirus (CMV) protein pp65.

T cell receptor (TCR) gene therapy is a promising immunotherapeutic strategy to treat a variety of virus- and cancer-related indications. One key obstacle is the lack of availability of potent TCRs to be analyzed in preclinical and clinical settings. Current methods to detect and isolate antigen-specific TCRs have technical limitations, are restricted to certain MHCs or require prior knowledge of the antigenic epitope. Choice of target antigen is another key factor for the success of immunotherapies. Targeting antigens, which are specifically expressed in tumor tissue but not in normal tissue, are of particular interest.

Adoptive T cell therapies are based on the *ex vivo* activation and expansion of T cells to generate antigen-specific effector T cells for reinfusion into the patient (1). This process may be accompanied by genetic modification of T cells with new antigen receptors. Rapid *ex vivo* expansion protocols enable the generation of high numbers of T cells to break immune suppressive barriers of the tumor microenvironment to clear target cells. Overcoming immune suppressive barriers may lead to the uptake of lysed tumor cells by APCs and the presentation of further antigens – a process called epitope spreading. This may be followed by *de novo* priming of autologous T cells and reactivation of anergic T cells amplifying the anti-tumor immune response, which was induced by the adoptive T cell therapy. Adoptive T cell therapies using genetically unmodified as well as antigen receptor-engineered T cells have been used successfully to treat cancer and virus-associated diseases, refractory to other treatments (2).

A second strategy to provide T cell immunity to a patient is the application of a vaccine. Vaccines provide acquired immunity to particular pathogens or cancers and market introduction of many vaccines have led to impressive declines in morbidity and mortality caused by numerous life-threatening diseases. For the development of novel prophylactic and therapeutic vaccines it is a prerequisite to identify antigens, epitopes and MHC restriction elements, which induce T cell responses that can provide protection from and clearance of pathogenic cells expressing such antigens. Identification of antigenic epitopes, which are endogenously processed and presented by pathogenic cells and which are recognized by antigen-specific TCRs enables the development of novel vaccine formulations to induce antigen-specific immunity.

In the following, current methods to identify and isolate antigen-specific TCRs and to identify antigenic epitopes, which induce T cell responses, are reviewed.

Peripheral blood mononuclear cells (PBMCs) are the primary source of T cells for *in vitro* analysis. For screening, large amounts of T cells can be isolated readily from PBMCs of patient or healthy donor blood. In comparison, T cells isolated from tumor-infiltrating lymphocytes (TILs) are available in limited amounts from tumor biopsies. Biopsies can only be obtained from solid tumors (3,4). Direct sequence analysis of TCRs from PBMCs or TILs can generate massive data on TCR sequences. Prediction of TCR specificity and antigen recognition from these data sets remains impossible (5). To isolate TCRs with desired specificity from T cell samples, it is necessary to introduce an antigen-specific enrichment step before analyzing TCR sequences. Additionally, it is useful to perform functional assays to confirm antigen specificity of T cells before TCR isolation (6). Standard approaches to enrich T cells are based on antigen-specific *in vitro* stimulation to expand specific T cells in culture. One strategy of *in vitro* stimulation of antigen-specific T cells is the addition of peptides to the T cell culture, which bind to MHC I molecules. Cells in culture present the peptides to each other and antigen-specific $CD8^{+}$ T cells grow out. However, peptide stimulations, which is often performed at non-physiological concentrations, may lead to overstimulation and activation-induced cell death of T cells harboring high affinity TCRs to peptide/MHC (pMHC) I (7). Additionally, knowledge of epitope sequence, processing properties and MHC I binding restriction is required. Furthermore, presence of peptide-specific T cells within PBMC or TIL samples is necessary. Thus, screening for peptide-specific T cells with low precursor frequency may be limited and precludes identification of novel immunogenic antigen-MHC I combinations. Functional testing of T cells for antigen-specificity and MHC restriction is usually carried out with single T cell clones. After expansion and enrichment, single T cell clones can be grown to sufficient amounts. One factor, which must be taken into account when applying T cell cultures, is the influence of long-term cultivation conditions on resulting T cells (8). Growth capacity of T cells with a more differentiated phenotype (Tem and Teff) is limited and restricts *in vitro* expansion. Long-term cultivation might lead to the loss of potent TCRs of T cell clones, which have already encountered pMHC I *in vivo* and undergone

expansion and differentiation in the donor. Thus long-term T cell cultures may introduce a bias in favoring outgrowth of undifferentiated T cells with high expansion capacity.

Detection and sorting of antigen-specific T cells is a critical step for the isolation of novel antigen-specific TCRs. The huge repertoire of TCRs with distinct specificities for pMHC led to the development of soluble MHC multimers as reagents to stain T cells specific for a known pMHC (9–11). Multimers are used for sorting of antigen-specific T cells. Multimers enable direct staining of T cells specific for the desired pMHC (12,13). However, prior knowledge of MHC restriction and peptide specificity is required to screen antigen-specific T cells within a sample. Use of multimers may rely on *in silico* prediction of T cell epitopes and thus harbors the risk to isolate TCRs specific for epitopes, which are not endogenously processed and presented. Additionally, T cells are selected upon their capability to bind multimer, but not necessarily upon binding to a pMHC complex at the cell surface. Multimers are custom-made reagents, which are not readily available to screen sets of different pMHCs.

In 1997, the groups of Lemonnier and Pérarnau reported the generation of a transgenic mouse stably expressing a single-chain construct of the human HLA-A*02:01 molecule fused to β-2 microglobulin (b-2m) (HHD). Additionally, mice were knockout for murine MHC I genes. Thus, immunization results in antigen-specific T cells exclusively restricted to HHD (14). HLA-transgenic mice have since provided a novel source of antigen-specific T cells (15). Human antigens and epitopes, which are not similar in their sequence to murine counterparts, can be used for vaccination of HLA-transgenic mice to elicit antigen-specific T cell responses in the non-tolerant repertoire, because mouse T cells were not deleted in the thymus and recognize human antigens as foreign. HLA-transgenic mice provide a source of high affinity TCRs recognizing self- or similar-to-self-antigens, which have been deleted in the human repertoire due to thymic selection. Technological advances were made, when based on the HHD mouse model the first transgenic mouse was generated carrying the human TCR repertoire while in parallel the murine TCR locus was knocked out (16). However, potential problems using MHC-transgenic and/or TCR locus-transgenic mice may emerge, e.g., it is difficult to predict whether or not mouse and human thymus selection follows the same rules. Transgenic MHC molecules are artificial single-chain constructs, which may introduce a bias to selecting TCRs functionally restricted to single-chain MHCs but not native MHC I complex. Immunization of single MHC-transgenic mice does not reflect a natural immune response with a choice of six cognate MHC:I complexes. It is likely that antigens exist, which do not contain e.g. HLA-A*02:01 epitopes. Furthermore, mice carrying the human TCR locus lack few TCRs chains, which may be crucial for targeting certain pMHC I (16). In contrast, immunization of mice carrying the murine TCR locus will result in the isolation of murine TCR sequences, which may be prone to rejection when transferred into humans (17,18). An important economical factor is the infrastructure necessary to work with murine models, which is very resource-intensive with regard to costs, regulatory requirements and manpower.

Successful TCR gene therapy is dependent on the availability of novel TCRs with exquisite specificity profiles. In light of this, the present inventors addressed the need to provide a novel method to identify and isolate novel antigen-specific TCRs, and to define epitope specificity and MHC restriction of these TCRs. For the development of novel vaccines, which induce antigen-specific T cell responses, there is a need for defining novel immunogenic epitopes, which can be targeted by T cells.

This problem is solved by the present invention, in particular, by the subject matter of the claims.

The present invention provides a method for preparing a nucleic acid encoding the TCR alpha chain construct (TRA) and TCR beta chain construct (TRB) of a TCR construct specific for an epitope from a defined antigen presented on an MHC, comprising:

(a) stimulating T cells isolated from a donor with professional antigen presenting cells (APCs) presenting epitopes of said defined antigen, to enrich antigen-specific T cells; and
(b) contacting said T cells with a library of cells, wherein each cell expresses a single MHC allele, wherein the library comprises cells expressing all MHC I or MHC II alleles present in the donor, and wherein the cells of said library present epitopes of said defined antigen; and
(c) selecting T cells activated by said contact, preferably, based on an activation marker expressed by said activated T cells; and
(d) isolating the nucleic acids encoding the TCR alpha and TCR beta chain of the TCR of said T cells.

In the context of the present invention “a” does not exclusively refer to “one”, but also encompasses “two or more”. For example, the method of the present invention can be used to prepare one or more, e.g., two, separate nucleic acids encoding the TRA and TRB of a TCR construct.

The term “capable of specifically binding” or “recognizing” or “specific for” a given antigen, as used herein, means that the TCR construct can specifically bind to and immunologically recognize said epitope, preferably with high affinity. Affinity can be analyzed by methods well known to the skilled person, e.g. by BiaCore.

One of the advantages of the present invention is that it is not required that immunogenic epitopes from the antigen presented on a specific MHC allele are known. With the method of the invention, it is possible to analyze T cell responses to antigens, where epitopes are naturally processed and presentation may occur via each possible MHC. Thus, the method of the invention may be used to identify a TCR specific for a defined antigen without prior knowledge of the epitope. The defined antigen may even be one of a plurality of antigens, e.g., a plurality of antigens of a specific virus, or all antigens of a specific virus, if both the antigen presenting cells in step a and the library in step b present epitopes of said antigens.

A further advantage is that the method of the invention renders it possible to identify TCRs specific for immunodominant peptide epitopes, and to provide said peptide epitopes. It has been observed that certain peptide-MHC (pMHC) combinations induce a dominant T cell response over other pMHC combinations derived from the same antigen even though both combinations are predicted to have high binding affinities. Additionally, this method may also enable the isolation of TCRs targeting subdominant epitopes, if the number of PBMCs screened is largely increased.

As therapy of humans is of most interest, it is preferred that the method is carried out in the human system, i.e., the donor is human, and, accordingly, human MHC I or MHC II, in particular, MHC I molecules, and APCs are used. It is of course also possible to use T cells from a murine donor, which are restricted to human MHC molecules (14,15) or murine T cells expressing human T cell receptors (16). Alternatively, other, e.g., completely murine, rat, goat, rabbit, guinea pig systems may be used if provision of a TCR from that species is desired.

Preferably, the T cells stimulated in step a are in the form of PBMCs, i.e., not separated from other mononuclear cells. This may be beneficial because it provides a more natural environment and excludes the need for additional purification steps. TILs isolated from a donor may also be used. Alternatively, purified T cells, or purified $CD4^+$ or, preferably, $CD8^+$ T cells may be used.

The professional APCs are preferably autologous APCs, i.e., they are isolated from the same donor as the PBMCs. They can however also be heterologous APCs, as long as they share at least one MHC allele, preferably, all MHC I or II (preferably MHC I) alleles with the donor. Most preferably, the professional APCs are dendritic cells, in particular, mature dendritic cells. The professional APCs present epitopes of the defined antigen on the MHC after endogenous processing of the antigen. The antigen is preferably provided to the inside of the professional APCs as RNA, DNA, protein or polypeptide to enable endogenous processing and presentation by the APCs. Therefore, prior knowledge of the epitope is not required. For example, stable or transient expression after transfection is possible.

The stimulation of T cells in step a is carried out for 7-42 days, most preferably for 14-28 days. The ratio of PBMCs to professional APCs preferably is about 5:1 to 20:1, most preferably, about 10:1. T cells are stimulated in medium containing cytokines favoring T cell proliferation; preferably low concentrations of IL-2 (e.g., 15-25 U/ml, preferably, about 20 U/ml), IL-7 (e.g., 2.5-7.5 U/ml, preferably, about 5 U/ml) or IL-15 to prevent antigen-unspecific T cell proliferation in the specific culture settings. Proliferating PBMCs may be split at ratios of about 1:2 to 3:4. A second stimulation with professional APC presenting an epitope of the antigen after endogenous processing is possible.

The cells enriched for antigen-specific T cells in step a are further contacted with a library of cells in step b, wherein each cell expresses a single MHC allele, wherein the library comprises cells expressing all

MHC I or II alleles present in the donor, and wherein the cells of said library present epitopes of said defined antigen.

It is preferred throughout the invention that the MHC is MHC I. The T cells stimulated by these cells, which express MHC I, will be $CD8^+$ T cells, and, typically, epitopes from intracellular proteins will be presented.

In a preferred embodiment, the library consists of K562 cells stably expressing one MHC I allele each. Exemplary K562 libraries are described below or disclosed by Zeng et al. (19). Cells of the library may be of other origin than K562 cells, comprising any human or non-human APCs, e.g., lymphoblastoid cell lines (LCL) or NIH/3T3 cells.

The MHC cell library used herein was generated by stable transduction of the human K562 cell line (20,21) with single human MHC I alleles (HLA-A, -B and -C). K562 cells are of human erythroleukemic origin and lack expression of endogenous MHC I and MHC II alleles (22). However, they express β-2 microglobulin, and upon transgenic expression of an MHC I α chain they possess a fully functional antigen-processing and presentation machinery (19,23,24). K562 cells express ICAM-1 and LFA-3, which are needed to form an effective immune synapse (24). Furthermore, it was possible to genetically modify these cells e.g. via retroviral transduction to stably express single MHC I alleles and to introduce antigenic sequences e.g. via transfection with *in vitro* transcribed (ivt) RNA.

In one embodiment, the present invention thus also provides a K562-based MHC cell library, cells of which may be employed in the method of the invention. Said library comprises K562 cells, each cell expressing one of the following MHC I alleles:

K562-based MHC cell library

HLA-A*	**HLA-B***	**HLA-C***
01:01	07:02	01:02
02:01	07:04	02:02
03:01	08:01	03:03
03:05	15:01	03:04
11:01	18:01	04:01
23:01	27:05	05:01
24:02	35:01	06:02
26:01	35:08	07:01
29:02	38:01	07:02
31:01	40:01	12:03
33:01	41:02	15:02
66:01	44:02	16:01
68:02	44:03	16:02
68:24	47:01	17:01
	49:01	
	51:01	

56:01
57:01
57:03
58:01

Using K562 cells as artificial APCs scaffold has several advantages. Compared to LCLs, K562 cells lack viral sequences from herpes viruses like EBV (25,26). This is an important feature of the K562-based APC system to avoid EBV-specific T cell activation during analysis of bulk T cell samples, which may naturally contain EBV-specific T cells. Compared to acellular artificial APCs, in a cellular APC system pMHC presentation occurs at physiological levels at the cell surface of an intact cell membrane, which resembles the most native environment for TCR binding. Dominant expression of one MHC minimizes allo-recognition of TCRs. The capability of MHC-transduced K562 to present pMHC at the cell surface shows that antigen processing and MHC expression in parental K562 is not a general defect, but due to silencing of the endogenous MHC locus. K562 cells express a constitutive proteasome compared to immune proteasome-expressing DCs, which resembles antigen processing by a typical tumor cell (27). TCR-transduced T cells cocultured with K562 cells in the absence of the target antigen showed no background IFNγ release and CD137 up-regulation minimizing unspecific activation of T cells.

The attributes of K562 cells discussed above made them the preferred cellular artificial APC system to establish an MHC cell library as a target for the analysis of TCRs without prior knowledge of epitope specificity and MHC restriction. In principle, any target antigen may be expressed in K562 cells, which includes pathogen-derived antigens as well as tumor-associated antigens (TAA), cancer testis antigens (CTA) and lineage antigens. For cancer immunotherapy, tumor-specific antigens (TSA) are of particular interest to identify TCRs specific for viral epitopes or mutation-derived epitopes.

Latouche and Sadelain used plate adherent mouse fibroblast NIH/3T3 cells, which stably expressed single human MHC alleles (28) and thereby achieved endogenous presentation of epitopes in the context of human MHC complexes. NIH/3T3 cells have been successfully used to expand CMV-specific T cell lines for use in post-transplant adoptive T cell therapy (29), and may be used as an alternative library in the context of the invention.

Alternatively, the MHC may be MHC II. In that case, the library of cells preferably comprises MHC II expressing cells, such as K562 cells transfected with one MHC II allele each. An exemplary library is disclosed by Butler et al. (30). Alternatively, single MHC II-expressing cell libraries may be used which are based on human RM3 (Raji) B cells, which were generated following random mutagenesis with ethane methylsulfonate (EMS) to knock out the MHC II locus (31). The T cells stimulated by these cells

will be $CD4^+$, and, typically, epitopes from secreted proteins, proteins of different cell compartments, membrane proteins or cross-presented proteins will be presented.

The cells of the library, in particular K562 cells, may further express costimulatory molecules, e.g., CD40, CD40L, CD70, CD80, CD83, CD86, ICOSL, GITRL, CD137L and/or CD252, so that the cells can be tailored for the optimal contacting of T cell subsets. Sets of costimulatory molecules may be CD80, CD86 and CD137L, or CD80, CD83, CD64 (30), or CD80, CD70 and CD137L (19). This may amplify the T cell response to clearly detect antigen-specific IFNγ release and CD137 expression and may lead to the detection of a wider range of TCRs with regard to affinity. However, to isolate high affinity TCRs, it may be preferably if the cells of the library, in particular the K562 cells, do not express any further costimulatory molecules. Furthermore, cells of the library, in particular K562 cells, may express molecules, which enhance antigen processing and presentation, e.g. HLA-DM and CD74.

The cells of the library present epitopes of the defined antigen on their MHC molecules after endogenous processing. Preferably, they stably express the complete full-length antigen, e.g., after transfection with antigen-encoding ivtRNA. Transient expression may also be used. Accordingly, prior knowledge of the epitope is not required.

Contacting of the library with T cells is performed between 12 and 36 hours, preferably, 18-22 hours or about 20 hours to achieve optimal activation of antigen-specific T cells within the T cell sample. Preferably, the addition of cytokines is avoided during contacting to prevent T cells from unspecific activation.

Those T cells carrying a TCR specific for an epitope presented by the cells of the library are activated. Accordingly, they can be selected based on an activation marker (step c), so that prior knowledge of the T cell epitope and MHC restriction element is not required. T cells up-regulate activation markers upon engagement of the TCR with the cognate pMHC complex on target cells. TCR signaling together with costimulatory signals induces short-term expression of activation molecules like CD25, CD69, CD107, CD137 and/or CD154, which can be used as markers for detecting and isolating antigen-specific T cells (32), e.g., by FACS or MACS. CD137 was shown to be a specific marker for the isolation of antigen-specific $CD8^+$ T cells (33,34). Sorting based on CD137 expression, e.g., by FACS, is a preferred method for selection of specific T cells. In one embodiment, this is combined with measurement of IFNγ release e.g. by enzyme-linked immunosorbant assay (ELISA) or by cytokine capture of IFNγ by FACS. Measuring IFNγ release e.g. by ELISA is a standard method to evaluate functional activity of T cell samples.

Cytokine capture assays provide an alternative tool to detect and isolate functionally active T cells without prior knowledge of pMHC specificity (35,36). $CD8^+$ T cells activated through cognate pMHC I release vesicles with cytokines like IFNγ and TNFα. Detection and isolation of individual T cells upon cytokine secretion has been facilitated through the development of cytokine capture assays in

combination with FACS or MACS analysis (36,37). Cytokine capture assays provide an attractive alternative to T cell activation markers for the sorting of T cells, which were activated by TCR signaling.

After selection of activated T cells, the nucleic acids encoding the TCR alpha and TCR beta chain of the T cell receptor of said T cells are directly isolated. E.g., RNA may be isolated to generate cDNA via 5' rapid amplification of cDNA ends (RACE) of TCR alpha and TCR beta genes followed by PCR amplification. PCR products may be cloned into expression plasmid to transform bacteria. Each bacterial colony may be regarded as containing one sequencing vector with one PCR TCR alpha or TCR beta gene fragment. Vector DNA of numerous bacterial colonies may be prepared, followed by sequencing of vector inserts (TCR alpha or TCR beta gene fragments). Sequencing results of each bacterial colony may be analyzed, e.g., by using IMGT/V-Quest. Frequencies of identical TCR alpha or TCR beta chains reflect the proportion of identical T cell clonotypes within the sorted T cell sample. Another strategy to analyze TCR alpha and TCR beta gene sequences of sorted T cells is the use of next-generation sequencing approaches. For example, PCR products of TCR alpha and TCR beta genes, which were ligated into sequencing vectors may be transformed into bacteria and grown in flasks containing selective medium and followed by preparation of the vector DNA. Vector DNA preparations may be directly used for next-generation sequencing analysis. Frequency of vectors containing identical TCR alpha or TCR beta genes are regarded to be representative for the initial amount of T cells of the same clonotype within the sample of sorted T cells. Frequency matching of TCR alpha and TCR beta chains within a T cell sample can be used for analyzing pairing of functional TCRs (38), e.g., TCRs which had accounted for antigen-MHC-specific IFNγ release and CD137 upregulation. Sensitivity of these methods enables detailed analysis of TCR repertoires within T cell samples. Matching the frequencies of TCR alpha and TCR beta chains additionally enables the reconstitution of abundant TCR chain pairs from T cell samples, thus making resource-intensive T cell clone culture dispensable. Long-term cultivation is necessary to expand T cell clones in culture, which may only favor the outgrowth of T cells, which had a naïve or central memory phenotype, therefore excluding T cells from the analysis, which have limited growing capacity *in vitro*. For the analysis of TCR repertoires it is thus an advantage to screen and sort antigen-MHC-specific T cell responses after only 14 to 28 days of antigen-specific enrichment.

Thus, preferably, if after analysis of the nucleic acids encoding the TCR alpha and TCR beta chains of the selected population of T cells, more than one TCR alpha and TCR beta chain is identified, the frequency of the TCR alpha and TCR beta chains in the population is analyzed and TCR alpha and TCR beta chains constituting a TCR capable of recognizing the epitope are matched based on their frequency.

Alternatively, further analysis may be carried out, e.g., generation of T cells carrying combinations of different predominant TCR alpha and TCR beta chains, and analysis of their activation by cells of the library used in step b.

The TCR alpha and beta chains may be modified, leading to TCR alpha chain constructs (TRA) and TCR beta chain constructs (TRB) of the invention. Optionally, codon usage of the TRA and TRB may be optimized to enhance expression of the TCR in recombinant T cells. Furthermore, human variable regions may be combined with murine constant regions (39), or a minimal murine constant region, i.e., human constant regions containing only defined amino acids from the murine constant region (40) and additionally comprising an additional cysteine bridge (41,42), which increases preferential binding of transgenic TCR chains to each other and reduces pairing with endogenous TCR chains expressed by recipient T cells.

Further optimization of expression is possible, e.g., by generating single-chain TCR constructs harboring the variable regions of the TRA and TRB to avoid pairing of endogenous TCR chains with introduced chains and to enhance functional activity. Furthermore, soluble receptor molecules and fusion proteins may be generated containing the variable regions of the TRA and TRB chain genes and e.g. antibody domains.

T cells specific for an epitope from a defined antigen presented on an MHC may be generated by expressing the nucleic acids encoding the TRA and TRB. If such T cells are intended for therapy of a patient, it is preferred to use autologous T cells. Alternatively, an allogeneic setting is possible, using immune suppression.

Analysis of activation of T cells transfected with suitable TCR constructs with cells of the library expressing specific MHC alleles may easily be used to analyze MHC restriction of the TCR.

The present invention further provides a method for identifying an epitope of a defined antigen capable of being presented by an MHC, comprising carrying out steps a-d of the method of the invention described above, and identifying the epitope capable of activating said selected T cells, or T cells modified with nucleic acids encoding the TRA and TRB constituting the TCR construct. Epitope mapping strategies are known in the art. For example, cells of the library expressing the relevant MHC allele may be transfected with minigenes covering sections of the relevant antigen, and responses, e.g., IFNγ secretion, analyzed to find the section harboring the epitope. Peptide pulsing with external peptides may also be helpful. Epitope prediction may be employed as part of such a strategy, but it is important to note that, as shown by the experiments described below that TCR specificity not necessarily matches predicted data from epitope prediction algorithms.

Furthermore, the epitope identified to induce antigen-specific T cell responses, which is demonstrated by the isolation of antigen-specific TCRs accounting for this response, may be used to develop vaccine formulations containing the epitope sequence. The method described in the inventions ensures the recognition of epitopes on MHC, which are endogenously processed and presented by cells expressing a

defined antigen. Application of the epitope-containing vaccine may provide T cell immunity to patients to clear cells, which naturally express the defined antigen.

In summary, the method of the invention has several advantages over conventional methods of providing TCR constructs which may be used, e.g., for adoptive T cell therapy. For example, the full repertoire of T cells is covered, e.g. including all cognate MHC I restriction elements of a given donor. The method also allows for detection of T cells with desired antigen specificity, but does not require previous knowledge of epitopes. It is based on the recognition of endogenously processed and presented epitopes without predetermination of selected epitopes. Different epitopes from one antigen have an expression hierarchy defined as immune dominance. TCR constructs identified by the method of the invention recognize the immune dominant epitopes within target antigens, which are presented most efficiently on one of the six MHC I alleles. The method also allows for the identification of T cells recognizing subdominant epitopes from the same antigen. Finally, the method allows for parallel detection of different T cell clonotypes recognizing the same antigen.

The invention also provides novel TCR constructs and the epitopes recognized by these TCR constructs, which are obtainable by the method of the invention and/or disclosed below, as well as nucleic acids, e.g., vectors, such as expression vectors, encoding these TCR constructs or the respective TRA and TRB or fragments thereof, such as variable fragments or the CDR3 region of the TRA or TRB, or transgenic T cells expressing said nucleic acids.

Human papillomavirus (HPV) infection is the primary cause for the development of cervical cancers, anogenital cancers and head and neck squamous cell carcinomas (43,44). Oncogenic HPV types accounting for approximately 610 000 cancer cases per year worldwide.

The most prevalent oncogenic type is HPV16 accounting for over 50% of cervical cancer cases. HPV16 E6 and E7 have transforming potential and are regarded as driving oncogenes (45,46). HPV16 E5 has been shown to have oncogenic potential, because of its ability to transform fibroblasts *in vitro* (47,48). Furthermore, it was found that E5 is expressed in biopsies of invasive cervical cancer indicating an important role of E5 in initiating and maintaining the transformed phenotype of malignant keratinocytes (49–51).

While prophylactic vaccination programs together with medical screenings and interventions will reduce HPV-related morbidity in several countries over the next centuries, HPV will remain a significant global health issue with a need for therapeutic treatments of cervical cancer and other HPV-caused cancers.

Attempts of therapeutic treatment of HPV-associated malignancies have been reported in numerous studies. However, only one phase I/II study applying synthetic long peptides covering the complete HPV16 E6 and E7 sequence has shown data of patients, which experienced regression of high-grade vulvar intraepithelial neoplasias (VIN) in more than 50% of the patients. This response was associated with HPV-specific T cell responses (52). Nevertheless, comparable results targeting CIN lesions and cervical cancer are lacking, suggesting that current attempts of therapeutic vaccination targeting HPV16 E6 and E7 do not overcome immune escape mechanisms of HPV (52,53). Furthermore, potent TCRs targeting E6 and E7 are lacking, which allow validation of immunotherapeutic treatment of HPV-induced malignancies.

As further therapeutic strategies are sought to treat HPV-induced cancers, the inventors' efforts concentrated on isolation of antigen-specific TCRs for HPV-derived antigens and the identification of immunogenic epitopes from these antigens using the method described above. HPV16 E5, E6, E7 and L1 were used as antigens.

Cytomegalovirus (CMV), also designated human herpes virus 5 (HHV-5), is a member of the herpes virus family. CMV infections resemble other herpes virus infections as they are controlled by $CD8^+$ and $CD4^+$ T cells, which occur at very high precursor frequency in CMV-seropositive individuals (54). CMV infection is usually asymptomatic in immune competent individuals and persists lifelong in a latent stage in up to 90% of the population. CMV disease may manifest in some immune compromised individuals after birth or after hematopoietic stem cell transplantation or solid organ transplantation causing a total of 5,600 severe disease cases with 560 deaths per year in the USA (54). CMV has widely been regarded as non-oncogenic, as infection does not seem to lead to cell transformation, but may opportunistically infect malignant cells. Recent studies have associated CMV with glioblastoma, questioning that CMV is a non-oncogenic virus (55–58). In light of this, the inventors have decided to also investigate TCRs specific for CMV antigens.

A strong T cell response to HPV16 E5 in combination with HLA-B*15:01 was observed upon screening with antigen-expressing K562 cells of the MHC cell library. Additionally, a CMV pp65-specific T cell response was observed over HLA-B*07:02. T cell samples responding to one antigen-MHC combination were directly sorted and analyzed for TCR gene sequences. Predominant TCR genes in a sample were cloned into a retroviral expression vector and testing was assessed by transducing different TCR alpha and beta chain combinations into PBMCs. A functional HPV16 E5-specific TCR and a functional CMV pp65-specific TCR could be reconstituted, which recognized endogenously processed epitopes presented on HLA-B*15:01 and B*07:02, respectively.

In particular, the invention thus provides an HPV E5-specific TCR construct, which may be used, e.g., for TCR gene therapy to treat HPV infection, in particular, HPV-induced malignancies. The invention provides a nucleic acid encoding a TRA and/or TRB of a TCR construct specific for an epitope of human papillomavirus 16 oncoprotein E5 in complex with a human MHC I. The nucleic acid may be obtainable or obtained from the method of the invention described herein.

The inventors provide a TCR construct specific for an epitope of human papillomavirus 16 oncoprotein E5 in complex with HLA-B*15:01, which preferably comprises SEQ ID NO: 1. The TCR construct preferably also recognizes an N-terminally longer version of the epitope, e.g., it may consist of SEQ ID NO: 1, 45, 47, 49, 51, 52 and 57. The inventors showed for the first time that these peptides are endogenously processed and may be presented on HLA-B*15:01, and may thus be the target of TCR recognition. Besides TCR gene therapy, vaccines comprising peptides of SEQ ID NO: 1, 45, 47, 49, 51, 52 and 57 may be a second strategy to provide T cell immunity in HLA-B*15:01-positive patients. Around 12% of the German population carry at least one HLA-B*15:01 allele. Population studies in China, South Korea and Japan have been found to have even higher B*15:01 allele frequencies (59).

Specificity of a TCR or TCR construct is mainly defined by the CDR3 regions of the TCR. The TCR construct of the invention specific for the E5 epitope presented on HLA-B*15:01 comprises a TRA comprising a CDR3 of SEQ ID NO: 2 or a CDR3 having at least 84%, preferably, at least 92% sequence identity thereto, i.e. there may be one or two substitutions, insertions or deletions. If there are mutations, conservative substitutions are preferred. SEQ ID NO: 2 is a preferred sequence of a CDR3 of a TRA of the invention. In one embodiment, the TRA comprises the CDR3 of SEQ ID NO: 2, CDR1 of SEQ ID NO: 64 and CDR2 of SEQ ID NO: 65.

The variable region of the TRA preferably comprises SEQ ID NO: 3 or has a sequence identity of at least 80%, preferably, at least 90% or at least 95% to SEQ ID NO: 3, wherein, preferably, the variable region comprises SEQ ID NO: 2. The variable region may be encoded by a nucleic acid of SEQ ID NO: 60. The TRA preferably has a sequence identity of at least 80%, preferably, at least 90%, at least 95% or 100% to SEQ ID NO: 4 or consists thereof, wherein the constant region preferably is a murine constant region or a minimal murine constant region to enhance pairing of transgenic TCR chains. Alternatively, the constant region may also be human, which minimizes the risk for immune recognition by immune cells of the recipient.

A preferred, codon-optimized nucleic acid encoding the TRA of the TCR construct specific for the HPV E5 epitope of the invention is SEQ ID NO: 5.

The TCR construct of the invention specific for the E5 epitope presented on HLA-B*15:01 comprises a TRB comprising a CDR3 of SEQ ID NO: 6 or a CDR3 having at least having at least 84%, preferably,

92% sequence identity thereto, i.e. there may be one or two substitutions, insertions or deletions. Conservative substitutions are preferred. SEQ ID NO: 6 is a preferred sequence of a CDR3 of a TRB of the invention. In one embodiment, the TRB comprises the CDR3 of SEQ ID NO: 6, CDR1 of SEQ ID NO: 66 and CDR2 of SEQ ID NO: 67.

The variable region of the TRB preferably comprises SEQ ID NO: 7 or has a sequence identity of at least 80%, preferably, at least 90% or at least 95% to SEQ ID NO: 7, wherein, preferably, the variable region comprises SEQ ID NO: 6. The variable region may be encoded by a nucleic acid of SEQ ID NO: 61. The TRB preferably has a sequence identity of at least 80%, preferably, at least 90%, at least 95% or 100% to SEQ ID NO: 8 or consists thereof, wherein the constant region preferably is a murine constant region or a minimal murine constant region to enhance pairing of transgenic TCR chains. Alternatively, the constant region may also be human, which minimizes the risk for immune recognition. A preferred, codon-optimized nucleic acid encoding the TRB is SEQ ID NO: 9.

The invention also provides a nucleic acid encoding both TRA and TRB specific for the HPV E5 epitope in a suitable expression cassette, e.g., for TCR gene therapy to treat HPV infections, in particular, HPV-induced malignancies, e.g., comprising the sequence according to SEQ ID NO: 40.

Based on the sequences provided by the method of the invention, it is possible to carry out affinity maturation of the TCR sequences (60,61). Non-synonymous nucleotide substitutions which lead to amino acid exchanges in the CDR3 sequence may lead to enhanced affinity of the TCR to target antigen. Furthermore, TCR sequence changes in other parts of the variable TRA and TRB regions may change, preferably increase, affinity of the TCR construct to the pMHC complex. It is preferred that TCR constructs varying from the specific sequences provided retain exclusive specificity for the target antigen provided. Accordingly, it is preferred that adoptive transfer of T cells expressing the TCR construct of the invention has no significant negative effects on healthy tissue.

The invention further provides a CMV pp65-specific TCR construct, which may be used, e.g., for TCR gene therapy to treat CMV infection or CMV-induced malignancies. The invention provides a nucleic acid encoding a TRA and/or TRB of a TCR construct specific for an epitope of human CMV protein pp65 in complex with a human MHC I. The nucleic acid may be obtainable or obtained from the method of the invention described herein.

The inventors provide a TCR construct specific for an epitope of human CMV protein pp65 in complex with HLA-B*07:02, which preferably comprises SEQ ID NO: 10 or consists thereof. The CMV pp65-specific TCR is restricted to HLA-B*07:02. HLA-B*07:02 is one the most frequent HLA-B alleles and found in about 23% of the German population (59). Epitope recognition by T cells may be the basis for the development of CMV vaccines inducing T cell immunity in HLA-B*07:02-positive patients.

The TCR construct of the invention specific for this epitope/MHC complex comprises a TRA comprising a CDR3 of SEQ ID NO: 11, which accordingly is a preferred CDR3 of a TRA of the invention. In one embodiment, the TRA comprises the CDR3 of SEQ ID NO: 11, CDR1 of SEQ ID NO: 68 and CDR2 of SEQ ID NO: 69. The variable region of the TRA preferably comprises SEQ ID NO: 12 or has a sequence identity of at least 80%, preferably, at least 90% or at least 95% to SEQ ID NO: 12, wherein the variable region comprises SEQ ID NO: 11. The variable region of the TRA may be encoded by a nucleic acid according to SEQ ID NO: 62. The TRA preferably comprises SEQ ID NO: 13 or consists thereof, wherein the constant region is a murine constant region or a minimal murine constant region to enhance pairing of transgenic TCR chains. Alternatively, the constant region may also be human, which minimizes the risk for immune recognition. A preferred, codon-optimized nucleic acid encoding the TRA chain of the TCR specific for the CMV pp65 epitope of the invention is SEQ ID NO: 14.

The TCR construct of the invention specific for this epitope/MHC complex comprises a TRB comprising a CDR3 of SEQ ID NO: 15, which accordingly is a preferred CDR3 of a TRB of the invention. In one embodiment, the TRB comprises the CDR3 of SEQ ID NO: 15, CDR1 of SEQ ID NO: 70 and CDR2 of SEQ ID NO: 71. The variable region of the TRB preferably comprises SEQ ID NO: 16 or has a sequence identity of at least 80%, preferably, at least 90% or at least 95% to SEQ ID NO: 16, wherein, the variable region comprises SEQ ID NO: 15. The variable region of the TRA may be encoded by a nucleic acid according to SEQ ID NO: 63. The TRB preferably comprises SEQ ID NO: 17 or consists thereof, wherein the constant region is a murine constant region or a minimal murine constant region to enhance pairing of transgenic TCR chains. Alternatively, the constant region may also be human, which minimizes the risk for immune recognition. A preferred, codon-optimized nucleic acid encoding the TRB chain is SEQ ID NO: 18.

The invention also provides a nucleic acid encoding both TRA and TRB specific for the CMV pp65 epitope in a suitable expression cassette, e.g., for TCR gene therapy to treat CMV infections, in particular CMV disease and CMV-induced malignancies, e.g., comprising the sequence according to SEQ ID NO: 41.

The nucleic acids of the invention may be provided as vectors comprising the nucleic acids of the invention encoding the TRA and/or TRB of the invention, e.g., vectors in which the TRA and/or TRB are operably linked to a promoter suitable for expression in T cells, such as human T cells. Preferably, said promoter is a heterologous promoter, i.e., it is not linked to TCR alpha or beta genes in naturally occurring cells, in particular, in human T cells. The promoter may be a constitutive or inducible promoter, preferably a myeloproliferative sacrcoma virus (MPSV), a CMV, a CAG or an EF1α promoter. The nucleic acids of the invention may be provided as RNA, as retroviral vectors, as γ-retroviral vectors, as

lentiviral vectors or as transposon-based vectors. In one embodiment, the vector is suitable for TCR gene therapy of a human patient. Preferably, the vector is MP71. Nucleic acids of the invention, e.g., encoding TRA and TRB may be fused via a genetic linker, preferably a virus-derived 2A element, to provide a single transgene cassette encoding both chains of a functional TCR construct.

The invention also provides proteins encoded by the nucleic acids of the invention, or fusion proteins thereof, in particular TRA and/or TRB encoded by the nucleic acids of the invention and specific for the disclosed antigens, e.g., for the epitope of HPV16 oncoprotein E5. A TRA and/or TRB of the invention may comprise all characteristics or domains corresponding to its native counterpart, but this is not essential. Preferably, the TRA and TRB comprise at least a variable region, or a variable and a constant region, e.g., the variable and/or constant region having at least 80%, at least 90% or at least 95% sequence identity to a human variable or constant TCR region. For adoptive T cell therapy, it is preferred that the TCR construct is a TCR comprising full length TCR alpha and beta chains comprising variable, constant and transmembrane regions.

The construct may also be a fusion protein, for example, variable regions of the TCR chains may be fused to Ig domains, e.g., an IgG constant domain, preferably, anti-CD3 antibody domains in a fusion protein of the invention, e.g., to provide soluble monoclonal TCR reagents to target malignant cells expressing the respective pMHC at the cell surface and engaging T cells via e.g. an anti-CD3 targeting domain to provide effector functions to the target cells (63).

Single chain constructs (scTCR) are encompassed as well as heterodimeric TCR constructs. A scTCR can comprise a variable region of a first TCR chain construct (e.g., an alpha chain) and an entire (full-length) second TCR chain (e.g., a beta chain), or *vice versa*. Furthermore, the scTCR can optionally comprise one or more linkers which join the two or more polypeptides together. The linker can be, for instance, a peptide which joins together two single chains, as described herein. Also provided is such a scTCR of the invention, which is fused to a cytokine, e.g., a human cytokine, such as IL-2, IL-7 or IL-15.

The TCR construct according to the invention can also be provided in the form of a multimeric complex, comprising at least two scTCR molecules, wherein said scTCR molecules are each fused to at least one biotin moiety, and wherein said scTCRs are interconnected by biotin-strepavidin interaction to allow the formation of said multimeric complex. Also provided are multimeric complexes of a higher order, comprising more than two, e.g., four, scTCR of the invention.

The TCR construct of the invention can be modified to comprise a detectable label, such as, for instance, a radioisotope, a fluorophore (e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE)), an enzyme (e.g., alkaline phosphatase, horseradish peroxidase), and particles (e.g., gold particles or magnetic particles).

The invention also relates to a host cell, preferably, a $CD8^+$ T cell comprising a nucleic acid encoding the TRA and TRB of a TCR construct of the invention, in particular a TCR construct specific for an epitope of human cytomegalovirus protein pp65, or, preferably, for an epitope of human papillomavirus 16 oncoprotein E5, and expressing said TCR construct. The TRA and TRB are preferably expressed from a heterologous promotor, e.g., as described above. The T cell preferably is a human T cell. It may be isolated from a patient infected with the relevant virus, in particular, a patient suffering from a malignancy associated with said virus, such as cervical cancer, anogenital cancer and head and neck cancer in the case of HPV. The patient expresses the MHC to which the epitope recognized by the TCR is restricted. The T cell may be a central memory T cell or a naïve T cell.

The invention also relates to such a host cell, e.g., T cell, or a nucleic acid of the invention for use in medicine, e.g., in a pharmaceutical composition. Such a pharmaceutical composition may be used for treatment of a patient infected with

a) human papillomavirus 16, wherein the TCR is specific for an epitope of human papillomavirus 16 oncoprotein E5 and wherein the patient is HLA-B*15:01-positive; or
b) human cytomegalovirus, wherein the TCR is specific for an epitope of human cytomegalovirus protein pp65 and wherein the patient is HLA-B*07:02-positive.

For use in medicine, the TCR construct comprising both TRA and TRB is employed, either in nucleic acid, protein or T cell form. It may be helpful to screen HPV-infected patients for expression of the E5 antigen prior to therapy, and treat only those patients with E5 expression.

The pharmaceutical composition may also be for use in prevention of infection or in reducing infection with a pathogen such as a virus, e.g.,

a) human papillomavirus 16, wherein the TCR is specific for an epitope of human papillomavirus 16 oncoprotein E5 and wherein the patient is HLA-B*15:01-positive; or
b) human cytomegalovirus, wherein the TCR is specific for an epitope of human cytomegalovirus protein pp65 and wherein the patient is HLA-B*07:02-positive.

For example, pharmaceutical compositions wherein the TCR is specific for an epitope of human papillomavirus 16 oncoprotein E5 and wherein the patient is HLA-B*15:01-positive may be used for prevention of cervical cancer in a patient infected with HPV16, e.g., if premalignant high-grade lesions of the cervix are detected. Pharmaceutical compositions wherein the TCR is specific for an epitope of human cytomegalovirus protein pp65 and wherein the patient is HLA-B*07:02-positive may be used for treatment of a patient after transfer of hematopoietic stem cells to prevent CMV disease or reduce symptoms thereof in said patient.

The invention also teaches a method of treatment of a patient in need thereof (e.g., infected with a virus such as HPV16 or CMV, or suffering from a malignancy associated with said virus), or of reducing infection with a virus, or symptoms of said infection, comprising administering to said patient a suitable recombinant T cell, or a nucleic acid of the invention. Treatment or prevention of post transplantation CMV disease (64,65) is also possible with T cells expressing or nucleic acids encoding the TCR of the invention specific for the CMV pp65 epitope.

The invention also relates to a nucleic acid encoding a fragment of human papillomavirus 16 oncoprotein E5 having a length of up to 40 amino acids comprising an epitope, or a peptide fragment of human papillomavirus 16 oncoprotein E5 having a length of up to 40 amino acids comprising an epitope, wherein the epitope is capable of being recognized by the TCR construct of the T cell of the invention. Such E5 fragments may be advantageously used in vaccination, e.g., for synthetic long peptide vaccination. Preferably, the nucleic acid encodes an epitope, or the peptide consists of an epitope, which can be identified by the method of the invention for identification of an epitope. Preferably, the epitope is an epitope of human papillomavirus 16 oncoprotein E5 capable of being recognized by the TCR construct described above. The E5 epitope, which has been identified for the first time by the method of the invention, preferably comprises SEQ ID NO: 1 and is selected from the group consisting of SEQ ID NO: 1, 45, 47, 49, 51, 52 and 57.

The invention also relates to said nucleic acid encoding said E5 peptide fragment or said epitope, or to said peptide fragment or epitope for use in medicine, preferably, for preventing infection (or reducing infection) with human papillomavirus 16, for prevention of cervical cancer in a patient infected with HPV16, e.g., if premalignant high-grade lesions of the cervix are detected, or for treatment of HPV16 infection, in particular, a HPV16-induced malignancy. Vaccines providing said epitope may be for administration to subjects, e.g., patients, who are HLA-B*15:01-positive.

The invention is further illustrated by the examples below, which are intended to exemplify the invention, and not to limit its scope. All references cited herein are herewith fully incorporated. All embodiments of the invention disclosed herein can be combined.

Figure legends

Fig. 1 Retroviral HLA vectors. Scheme of the γ-retroviral vector MP71 carrying different HLA alleles fused to an (a) IRES-GFP or (b) IRES-CFP expression marker.

Fig. 2 MHC class I cell library. K562 cells were transduced with the retroviral vector MP71 carrying an (a) MHC-IRES-GFP or an (b) MHC-IRES-CFP cassette. Shown is a selection of FACS plots of 10 MHC transductions covering all MHC class I alleles of two T cell donors. GFP and CFP expression indicate transduction rates. MHC surface expression of GFP- or CFP-positive cells is shown by MHC class I antibody staining and indicated in percentages.

Fig. 3 Examples of stable antigen expression in the MHC cell library. K562 cells were stably transduced with antigen (HPV16 E7) fused to an IRES-mCherry marker and detected by flow cytometry measuring of mCherry expression. MHC I alleles are fused to IRES-GFP marker and expression is indicated by flow cytometric detection of GFP. Percentages of MHC-IRES-GFP/antigen-IRES-mCherry double positive cells are depicted in the FACS plots. (a) K562 cells of the MHC cell library were stably transduced with E7. (b) MHC (HLA-B*15:01) -transduced K562 cells were stably transduced with the truncated microgene constructs of E5, which were used for epitope mapping (nt, nucleotides).

Fig. 4 Expression of antigens from ivtRNA in target cells. Single MHC-transduced K562 cells expressing IRES-CFP marker were transfected via electroporation with ivtRNA encoding GFP. Expression of GFP was measured 5 h after electroporation and served as control for transfection efficiency.

Fig. 5 Induction of antigen-specific T cell response upon coculture with K562 cells of the MHC cell library. (a) TCR-B23 and (b) TCR-S51 were stably transduced into T cells, which were stained for CD8 and transgenic TRB expression and analyzed by flow cytometry. (c) TCR-transduced T cells were cocultured with K562-B*27:05 target cells 3 h after E7co ivtRNA transfection. CD137 expression was measured by flow cytometry. H_2O-transfected K562-B*27:05 target cells served as a negative control.

Fig. 6 mDCs express GFP from ivtRNA. mDCs were generated from plate-adherent monocytes. (a) Histograms of flow cytometry show mDCs expressing T cell activation molecules CD80, CD83 and CD86 (black lines) compared to isotype controls (grey areas). (b) Four to six hours after transfection with 15 µg antigen ivtRNA, expression of GFP was measured. Percentages of GFP^+ DCs are indicated.

Fig. 7 Screening for virus-specific T cells. T cells, which had been stimulated antigen-specifically with autologous mDCs, were cocultured with the six corresponding single MHC I-expressing K562 cells of the MHC cell library. (a) Supernatant of the coculture was tested for IFNγ release with ELISA. (b) Cells from the same well were analyzed by flow cytometry to determine percentages of $CD137^+$ of $CD8^+$ T cells.

Fig. 8 FACS-sorting of virus-specific T cells. (a) T cells, which showed reactivity to pp65 (**Fig. 7**b), were cocultured with pp65-transfected HLA-B*07:02-expressing K562 cells of the MHC cell library. (b) E5-reactive T cells (**Fig. 7**b) were cocultured with E5-transfected HLA-B*15:01-expressing K562 cells of the MHC cell library. Single lymphocytes of both cocultures were gated in the FSC/SSC and CD137-expressing cells of $CD8^{+}$ cells were FACS-sorted for subsequent TCR analysis.

Fig. 9 Expression and functional analysis of TCR alpha and TCR beta chain combinations. (a) TCR alpha and TCR beta chain combinations were retrovirally expressed in PBMCs of healthy donors. Transgenic TCR expression in T cells was measured by flow cytometry after antibody staining for CD8 and the murine constant TCR beta chain segment. Untransduced (ut) T cells were used as a negative control. Result is representative for two independent experiments with different PBMC donors. (b) T cells transduced with the different TCR alpha and TCR beta chain combinations were cocultured with antigen (HPV16 E5 or CMV pp65)-transfected K562-B*15:01 and K562-B*07:02 target cells, respectively. IFNγ release of TCR alpha / TCR beta -transduced T cells was measured by ELISA. Results are shown as mean +/- SEM of duplicates.

Fig. 10 Generation of TCR gene-modified PBMCs with optimized TCR transgene cassettes. Retroviral transduction of PBMCs with TCR transgene cassettes was performed. Transduction rates were assessed by antibody staining of the murine TRBC followed flow cytometric analysis. Results are representative for experiments with PBMCs from two different donors (ut, untransduced PBMCs).

Fig. 11 Mapping of the antigenic sequence of HPV16 E5 (a) Full-length E5 wild type (wt) (252 nt), E5 codon-optimized (co) (252 nt) and 3’ truncated minigene versions (63-189 nt) of E5wt were fused to an IRES-mCherry marker, cloned into the MP71 retroviral vector and expressed in K562-B*15:01 target cells. Indicated is the length of the gene sequences starting with A of the ATG start codon. (b) TCR E5-transduced T cells were cocultured for 18 h with K562-B*15:01 target cells carrying one of the E5 gene versions. IFNγ release was determined by ELISA. Results are shown as mean +/- SEM of duplicates.

Fig. 12 Epitope mapping of the HPV16 E5-specific TCR and the CMV pp65-specific TCR. (a) HPV16 E5 epitopes predicted by IEDB as potential epitopes presented on HLA-B*15:01 were clustered according to sequence similarities. The first row (p4-p17) (SEQ ID NOs: 1, 49-61) indicates the rank of peptides in the epitope prediction (**Table 2**). The second row (-mer) denotes the peptide length. (b) E5 TCR-transduced PBMCs were cocultured with peptide-pulsed K562-B*15:01 target cells and IFNγ release was determined by ELISA. Untransduced (ut) T cells were used as a negative control. (c) CMV pp65 peptide p1 (SEQ ID NO: 10) represents a previously described epitope of pp65 presented on HLA-B*07:02(66,67). (d) Pp65 TCR-transduced PMBCs were cocultured with pp65 p1-pulsed K562-B*07:02

target cells. IFNγ release was measured by ELISA. All ELISA results are shown as mean +/- SEM of duplicates.

Examples

1.1 Generation of an MHC vector library

Genes for common MHC I alleles were cloned into the γ-retroviral vector MP71 (68–70) to first generate an MHC vector library, to generate single-MHC-expressing K562 cells (23), which were used as artificial APCs comprising the MHC cell library. Allelic versions of HLA-A, -B or -C genes are highly polymorphic. Sequences are open access at the IMGT/HLA database. However, the 5' and 3' ends of different types have high sequence similarities, making it challenging to PCR amplify one specific HLA gene from a cells' cDNA. To overcome this problem, cDNA was generated from lymphoblastoid cells (LCL) obtained from the International Histocompatibility Workshop, which were homozygous for the desired HLA-A, -B and -C alleles to enable efficient gene amplification by PCR. Amplified HLA fragments were fused to an IRES-GFP or IRES-CFP expression marker and cloned into the retroviral expression vector MP71 (**Fig. 1**).

1.2 Generation of MHC cell library

The erythroleukemic cell line K562 (20) was used as artificial APC scaffold for the generation of the MHC cell library. K562 cells lack endogenous expression of MHC class I molecules though expressing β-2 microglobulin, one ubiquitous component of functional MHC complexes. However, upon transfection with an MHC class I α-chain allele, the cells can be shown to possess a functional antigen processing machinery with MHC surface expression, thereby making K562 an attractive scaffold for the generation of artificial APCs (19,23,24). Stable transduction of K562 cells with single HLA alleles was conducted using the MP71 retroviral vector-based HLA library. Production of retroviral supernatant in 293T packaging cells and transduction was performed as described (72) and resulted in GFP- or CFP-expressing populations, as was confirmed by flow cytometric analysis. Functional assembly and surface expression of MHC complexes was indicated by MHC class I antibody staining of GFP- or CFP-positive cell populations (**Fig. 2**). All HLA alleles transduced in K562 cells were expressed at the cell surface. For later analysis of isolated TCRs, panels of K562 cells were generated covering all of the six MHC class I alleles of the original T cell donor.

1.3 Antigen expression in the MHC cell library

To use K562 cells of the MHC cell library as artificial APCs, single-MHC-expressing K562 cells were transduced with the retroviral vector MP71 to stably express antigenic constructs in the context of a single

MHC allele. Retroviral transduction was performed as described (71). Antigen expression in K562 cells allowed for endogenous processing and presentation of epitopes in the context of single MHC alleles. Many antigens (HPV16 E5, E6 and L1, CMV pp65 and IE-1) could not be detected readily by intracellular FACS staining. Furthermore, antibodies were not available for truncated antigens of HPV16 E5 (minigene constructs), which were used for epitope mapping, as well as mutated nucleotide sequences. Thus, all antigens were fused to an IRES-mCherry marker to indirectly confirm expression by flow cytometry (**Fig. 3**).

A second strategy to express antigenic sequences in target cells was to transfect ivtRNA via electroporation. Therefore, antigen sequences were cloned into expression vectors to enable T7 promoter-dependent generation of ivtRNA and subsequent polyadenylation using mMessage mMachine and poly(A) kits from Ambion (Life Technologies). Electroporation of ivtRNA into K562 cells was performed with a BioRad GenePulser using an exponential electroporation protocol. Generally, ivtRNA encoding GFP was used as a control for electroporation efficiency. **Fig. 4** shows that HLA-transduced K562 cells expressed GFP after ivtRNA electroporation.

1.4 Induction of antigen-specific T cell response by target cells of MHC cell library

In the previous experiments, it was shown that HLA-transduced K562 cells of the MHC cell library express a defined antigen after retroviral transduction or after transfection with antigen ivtRNA. The next step was to test the capacity of the MHC cell library to endogenously process and present epitopes to induce antigen-specific T cell responses. It has been described that HLA antigen-specific stimulation of T cells via the TCR leads to the upregulation of the early activation marker CD137 (32–34).

For this, two well-characterized TCRs (B23, S51) were used, which were isolated from antigen-specific T cell clones, recognizing endogenously processed and presented epitope on HLA-B*27:05. PBMCs engineered to express the TCRs (**Fig. 5**a, b) were used to analyze the capacity of antigen-expressing K562 cells of the MHC cell library to activate antigen-specific T cells. T cell activation was measured by CD137 expression via flow cytometry. As shown in **Fig. 5**c, all TCR-engineered PBMCs expressed CD137 activation marker after 20 h of coculture with antigen ivtRNA-transfected K562-B*27:05 target cells. The amount of $CD8^+/CD137^+$ T cells was around 6% for TCR-B23-engineered T cells and 8% for TCR-S51-engineered T cells, which reflects the total amount of TCR-engineered/$CD8^+$ T cells (**Fig. 5**a, b) used for the coculture. In conclusion, K562 cells endogenously processed the antigenic epitope and presented it on the transgenic HLA-B*27:05, which led to the stimulation of all antigen-specific T cells in the sample as measured by CD137 expression. Additionally, CD137 expression correlated with antigen-specific IFNγ release of TCR-transduced T cells as measured by ELISA.

2.1 Antigen-specific expansion of T cells

In the following, the setup of a screening approach to detect and isolate TCRs with desired antigen specificity is described. It can be transferred to different antigens, e.g., from different viruses, or different tumor-specific antigens.

Therefore, DCs were generated and matured from plate adherent monocytes (72,73) using endotoxin-free medium. Maturation state of mature DCs (mDC) was confirmed by staining for T cell activation markers CD80, CD83 and CD86 as well as MHC II expression followed by flow cytometry (**Fig. 6**). Antigen ivtRNA was generated from six viral antigens (CMV pp65 and IE1, HPV16 L1, E5, E6 and E7), which represented full-length reference HPV16 and CMV wild type gene sequences as indicated in the open access UniProt database. mDCs were transfected with ivtRNA of antigens to ensure the presentation of naturally processed and presented epitopes at the cell surface (74). ivtRNA encoding GFP was used as transfection control. Expression was measured 4-6 h after transfection (**Fig. 6**). Antigen-expressing mDCs were used at a PBMC to DC ratio of 10:1. PBMC stimulation with DCs was performed using medium containing 10% human serum (74,75). IL-2 (20 U/ml) and IL-7 (5 ng/ml) were provided with the medium from day 2 of stimulation on to favor T cell proliferation. Three times a week IL-2 (20 U/ml) and IL-7 (5 ng/ml) was provided to the culture. Proliferating PBMCs were splitted at ratios of 1:2 to 3:4.

2.2 Screening for virus-specific T cells

A second stimulation was performed 14 days after the first round of stimulation using autologous mDCs expressing one of the six viral antigens. After 28 days, the 12 T cell cultures were screened for reactivity to specific antigen-MHC combinations employing the MHC cell library (**Fig. 2**). Cells of the MHC cell library were transfected with antigen ivtRNA via electroporation and each T cell culture raised against one antigen was screened for reactivity to the antigen in combination with one MHC type. Contacting of the library with T cells was performed preferably 18-22 h to achieve optimal activation of antigen-specific T cells within the T cell sample. The addition of cytokines was avoided during contacting to prevent T cells from unspecific activation. IFNγ release of antigen-specific T cells was measured by ELISA. Further, the T cells in the coculture were analyzed for expression of CD137 by flow cytometry (33).

T cells showed specific reactivity to pp65 and E5 in combination with HLA-B*07:02 and HLA-B*15:01, respectively. Antigen-MHC-specific T cell reactivity could be measured by release of IFNγ and upregulation of CD137 at the T cell surface. Thus, combining cytokine release ELISA and flow cytometric analysis of T cell activation marker represents a robust two-method read out system for detecting antigen-MHC-specific T cell responses.

2.3 Sorting of virus-specific T cells to analyze the TCR repertoire

T cells, which showed specific responses to one antigen-MHC combination in both assays, were selected for FACS sorting to analyze the TCR repertoire. T cells, which were expanded with CMV pp65, were cocultured with pp65-transfected K562-B*07:02 target cells, and $CD137^+$ T cells were sorted from the culture. Nearly half of the $CD8^+$ T cells were $CD137^+$ in this setting. Thirteen percent of $CD8^+$ T cells expressed CD137 upon coculture with HPV16 E5-transfected K562-B*15:01 target cells.

After sorting of antigen-MHC-specific T cells, RNA was isolated and cDNA was generated using the SMARTer RACE cDNA amplification kit (Clontech) for 5'-RACE PCR of TCR alpha and beta genes. The PCR amplification generated TCR alpha and beta gene fragments, which quantitatively represented the amount of each T cell clonotype in the FACS-sorted T cell sample. PCR products of TCR alpha and beta gene fragments were ligated into sequencing vectors using the TOPO® cloning system (Invitrogen, Life Technologies), transformed into bacteria and grown on plates containing selective medium. Each bacterial colony was regarded as containing one sequencing vector with one PCR TCR alpha or beta gene fragment. Vector DNA preparations of numerous bacterial colonies were followed by sequencing of vector inserts (TCR alpha or beta gene fragments). Sequencing results of each bacterial colony were analyzed by using the web-based IMGT/V-Quest. Frequencies of identical TCR alpha or beta chains reflected the proportion of identical T cell clonotypes within the FACS-sorted T cell sample. Next, frequency matching of TCR alpha and beta chains was performed to reconstitute functional TCRs, which had accounted for antigen-MHC-specific IFNγ release and CD137 upregulation.

TCR analysis revealed TRAV17 and TRAV38-2 chains each to be present in nearly 40% of all T cells sorted upon response to HPV16 E5 and HLA-B*15:01. Three TCR beta variable chains were found to be present in 21-32% of T cells (**Table 1**). It was assumed that each of the two TCR alpha chains could assemble a functional E5-specific TCR with one of the three TCR beta chains. Thus, there were six possible combinations of candidate TCR alpha and TCR beta chains to assemble a functional HPV16 E5-specific TCR.

T cells sorted for reactivity to CMV pp65 and HLA-B*07:02 had one predominant TCR with a TRAV17 and a TRBV7-9 chain, both being present in about 70% of TCR alpha and TCR beta colonies, respectively (**Table 1**).

In sum, TCR analysis showed that CD137 sorting of antigen-MHC-specific T cells is accompanied by a strong enrichment for few predominant TCR alpha and beta chains, which appear at high frequency and which may reconstitute functional antigen-MHC-specific TCRs.

Table 1 TCR analysis

MHC class I	V segment	CDR-3	SEQ ID NO:	Frequency	% of total
Antigen HPV16E5					
HLA-B*15:01	Homsap TRAV17*01 F	CAESEYGNKLVF	2	10	38,5
	Homsap TRAV38-2/DV8*01 F	CAYRSWNYGQNFVF	19	10	38,5
	Homsap TRAV8-6*02 F	CAVSEPAAGNKLTF	20	2	7,7
	Homsap TRAV26-2*01 F	CILRGAGGTSYGKLTF	21	1	3,8
	Homsap TRAV8-6*02 F	CAVITNAGKSTF	22	1	3,8
	Homsap TRAV25*01 F	CAGPPSGTYKYIF	23	1	3,8
	Homsap TRAV6*02 (F)	CALPMEYGNKLVF	24	1	3,8
	Homsap TRBV5-1*01 F	CASSSRGHQNTGELFF	25	6	21,4
	Homsap TRBV12-3*01 F	CASSPEGEGVTGELFF	26	8	28,6
	Homsap TRBV6-5*01 F	CASSYRQQETQYF	6	9	32,1
	Homsap TRBV5-1*01 F	CASTLRGYTEAFF	27	1	3,6
	Homsap TRBV5-5*02 (F)	CASSPWADSNQPQHF	28	1	3,6
	Homsap TRBV20-1*01 F	CSAGTSGGPAYEQYF	29	1	3,6
	Homsap TRBV27*01 F	CASSSPLADDYNEQFF	30	1	3,6
	Homsap TRBV6-2*01 F ...	CASSHRRAHRAREQYF	31	1	3,6
Antigen CMV pp65					
HLA-B*07:02	Homsap TRAV14/DV4*01 F	CAMREGKDSSYKLIF	32	2	8,7
	Homsap TRAV17*01 F	CATVIRMDSSYKLIF	11	17	73,9
	Homsap TRAV8-1*01 F	CAVNRGGSNYKLTF	33	1	4,3
	Homsap TRAV3*01 F	CAVRDIGGFKTIF	34	1	4,3
	Homsap TRAV1-2*01 F	CALDGQKLLF	35	1	4,3
	Homsap TRAV4*01 F	CLVGGLRGNVLHC	36	1	4,3
	Homsap TRBV7-9*03 F	CASSLIGVSSYNEQFF	15	13	68,4
	Homsap TRBV27*01 F	CASRLGGGNYNEQFF	37	4	21,1
	Homsap TRBV20-1*01 F	CSASPRDRKFSGNTIYF	38	1	5,3
	Homsap TRBV7-9*03 F	CASSSHDNQGAKSPLHF	39	1	5,3

TCR alpha and TCR beta chains were amplified with TRAC- and TRBC-specific reverse primers from 5'-RACE cDNA of T cells, which had shown antigen-MHC-specific T cell responses. TCR analysis was performed with IMGT/V-Quest. V(D)J gene usage and CDR3 sequences specify identical TCR alpha or TCR beta chains. Frequencies of colonies carrying one TCR alpha or TCR beta chain are indicated. Percentage of total indicates the proportion of colonies with identical TCR alpha or TCR beta chains.

2.4 Functional analysis of TCR alpha and TCR beta chain combinations

For transgenic expression of TCRs, each TCR alpha and TCR beta chain gene was cloned into the γ-retroviral vector MP71. Cell surface expression and functional analysis of TCRs was performed after stable transduction of PBMCs with different TCR alpha and TCR beta gene combinations.

Variable regions of predominant TCR alpha and TCR beta chain genes (TRAV and TRBV), which are responsible for peptide-MHC (pMHC) I binding, were fused to codon-optimized murine constant TCR alpha and TCR beta gene segments (mTRAC and mTRBC) to enable preferential pairing of transgenic TCR chains after retroviral transduction of T cells (39,40,71). Staining of transgenic TCRs with an

antibody specific for the mTRBC was followed by flow cytometric analysis and showed that all TRA/TRB chain combinations were expressed in PBMCs (**Fig. 9**a). However, transduction rates of different combinations varied between 6-25%. For functional analysis, TCR-transduced T cells were tested for reactivity to K562-B*15:01 or K562-B*07:02 target cells, which were transfected via electroporation with HPV16 E5 or CMV pp65 antigen ivtRNA, respectively. Strikingly, T cells expressing TRAV17 (SEQ ID NO: 3) in combination with TRBV6-5 (SEQ ID NO: 7) recognized E5-transfected target cells, whereas none of the other five TRA/TRB combinations showed antigen-specific reactivity to E5 (**Fig. 9**b). Therefore, TRAV17 in combination with TRBV6-5 reconstituted a functional antigen-specific TCR. The only candidate TRA/TRB combination of CMV-reactive T cells (TRAV17 (SEQ ID NO: 12) and TRBV7-9 (SEQ ID NO: 16)) showed pp65-specific recognition of K562-B*07:02 target cells. TRA/TRB-transduced T cells showed no background reactivity to H_2O-transfected target cells (**Fig. 9**b), thus allowing clear identification of functional antigen-MHC-specific TCRs.

In conclusion, reconstitutions of TCRs from antigen-specific T cell clonotypes were achieved through the combination of TRA and TRB chains, which were found at high frequencies in FACS-sorted T cell samples. Screening, detection and isolation of TCRs with desired antigen specificity could be achieved by this approach, which was based on the use of the MHC cell library.

2.5 Optimization of HPV- and CMV-specific TCRs for transgenic expression in PBMCs

To increase efficiency of transgenic TCR expression, several optimizations of TCR transgene sequences were applied (71). TRA and TRB chain sequences were codon-optimized and human TRAC and TRBC gene segments were replaced by their murine counterparts to increase preferential binding of transgenic TCR chains and to reduce pairing with endogenous TCR chains expressed by recipient T cells (SEQ ID NO: 5, 9, 14, 18). The optimized TRB gene was then linked via a P2A element to the TRA genes and resulting single TCR transgene cassettes (E5-specific: SEQ ID NO: 40, pp65-specific: SEQ ID NO: 41) were molecularly cloned into the γ-retroviral vector MP71. Retroviral particles carrying the optimized TCR transgene cassettes were generated via a three-plasmid transfection of 293T cells and donor PBMCs were stably transduced with retroviral particles encoding the TCRs (70). TCR gene-modified T cells within the PBMC sample were analyzed by flow cytometry after antibody staining of the transgenic murine TRBC. TCR transduction rates of 45% for the E5-specific TCR (TRAV17 + TRBV6-5, SEQ ID NO: 40) and 37% for pp65-specific TCR (TRAV17 + TRBV7-9, SEQ ID NO: 41) could be achieved in PBMCs, whereby 27% and 22%, respectively, were positive for CD8 and the transgenic TCR. In conclusion, transgenic TCR expression could be improved markedly from 6-7% when using the non-optimized TRA and TRB single chain transgene cassettes, which were used to reconstitute functional TCRs, to approximately 40% when using the optimized TCR transgene cassettes.

2.6 Epitope mapping of the HPV16 E5-specific TCR

After detection of T cell clonotypes recognizing immunogenic antigen-MHC combination without prior knowledge of immunogenic epitopes, epitope mapping was performed to reveal the exact peptide sequence within the antigenic HPV16 E5 protein, which is recognized by the E5-specific TCR. The HPV16 E5-specific TCR composed of TRAV17 and TRBV6-5 sequences was an unique TCR, which has not been described before. To map the antigenic sequence recognized by the TCR, 3'-truncated minigene versions of HPV16 E5 generated by PCR with primers amplifying the respective gene region of interest. E5 minigenes were cloned into the retroviral vector MP71 and stably transduced into K562-B*15:01 target cells (**Fig. 11**a). T cells transduced with the optimized E5-specific TCR gene cassette recognized target cells carrying the full-length E5 and the 189 nt E5 minigene sequence but not 126 and 63 nt E5 minigenes as indicated by IFNγ release (**Fig. 11**b). Furthermore, E5 TCR-transduced T cells released IFNγ irrespective of whether the target cells harbored E5wt or E5co gene sequences (**Fig. 11**b). In conclusion, the HPV16 E5 TCR was specific for an epitope within the E5 protein region between amino acid 42-63.

To narrow down candidate epitopes that may be the target of E5-specific TCR recognition, *in silico* epitope prediction was performed using the web-based IEDB T cell epitope combined predictor, which integrates predictions of proteasomal cleavage, TAP transport, ER processing and MHC class I binding. Integrated epitope prediction was performed including 8-14-mer peptides in one analysis. Epitope prediction results were calculated as total score, which can be interpreted as the probability of a given peptide to be processed and presented on an MHC molecule at the cell surface. **Table 2** includes all epitopes (p1-p17) of the prediction with a positive total score using constitutive proteasome prediction. In contrast to DCs, K562 cells express a constitutive proteasome, which resembles proteasomes expressed in tumor cells (76). All epitopes, which were not expressed from amino acid sequence 42-63 of HPV16 E5 were excluded from further analysis. Surprisingly, the top three predicted epitopes (p1-p3) had to be discarded.

Table 2 Epitope prediction of HPV16 E5

#	Pos.	-mer	Sequence	Affinity to MHC [nM]	Total score	SEQ ID NO
p1	28	12	LIRPLLLSVSTY	42,98	0,70	42
p2	27	13	LLIRPLLLSVSTY	39,49	0,67	43
p3	72	9	FLIHTHARF	32,60	0,60	44
p4	***55***	***9***	***SAFRCFIVY***	***97,77***	***0,60***	***1***
p5	***54***	***10***	***ASAFRCFIVY***	***102,30***	***0,58***	***45***
p6	**48**	**10**	**LLWITAASAF**	**39,05**	**0,58**	**46**
p7	***51***	***13***	***ITAASAFRCFIVY***	***79,80***	***0,51***	***47***
p8	32	8	LLLSVSTY	63,70	0,38	48
p9	***53***	***11***	***AASAFRCFIVY***	***143,79***	***0,36***	***49***
p10	55	14	SAFRCFIVYIIFVY	138,79	0,36	50

p11	***50***	***14***	***WITAASAFRCFIVY***	***144,35***	***0,32***	***51***
p12	***52***	***12***	***TAASAFRCFIVY***	***149,57***	***0,28***	***52***
p13	**47**	**11**	**LLLWITAASAF**	**70,87**	**0,25**	**53**
p14	11	9	LLACFLLCF	119,99	0,10	54
p15	**50**	**8**	**WITAASAF**	**108,63**	**0,08**	**55**
p16	73	8	LIHTHARF	116,82	0,05	56
p17	***48***	***13***	***LLWITAASAFRCF***	***169,11***	***0,00***	***57***

Integrated MHC class I epitope prediction (IEDB) was used to rank the likelihood of candidate target epitopes (p1-17) for the HPV16 E5-specific TCR. Depicted is the position of the first amino acid of predicted peptides within the full-length HPV16 E5 protein (Pos.), peptide length (-mer), amino acid sequence, predicted binding affinity to HLA-B*15:01 and the SEQ ID NO. The algorithm uses a combined total score (arbitrary units), which integrates predictions for proteasomal cleavage, TAP transport and MHC (HLA-B*15:01) binding affinity. The table shows only peptides with a total score higher than zero. The higher the total score, the higher the efficiency of a peptide to be processed and presented at the cell surface. Epitopes in bold print were translated from 126-189 nt sequence of E5. Epitopes in italics were recognized by the TCR.

The result of the integrated epitope prediction of HPV16 E5 is listed in **Table 2**. The best predicted epitope was ranked first. Peptides encoded within amino acid sequence 42-63 are shown in bold print. These ten candidate epitopes were clustered according to sequence similarities (**Fig. 12**a) and peptides were exogenously loaded on K562-B*15:01 target cells. E5 TCR-transduced PBMCs specifically recognized HPV16 E5 epitope p4 (SAFRCFIVY, SEQ ID NO: 1). Additionally, the TCR clearly recognized all N-terminally elongated derivatives of SAFRCFIVY (SEQ ID NO: 1) including 12-, 13- and 14-mers conferring unconventional peptide lengths for MHC I (**Fig. 12**b). HLA-B*15:01 may tolerate binding of peptides which protrude at the N-terminus. In contrast, none of the other peptides was recognized, although peptide p6 and p13 were predicted to have higher binding affinities to HLA-B*15:01 than p4.

In sum, integrated epitope prediction facilitated mapping of the exact epitope recognized by the E5-specific TCR. However, the algorithm could not predict the immunogenic epitope, and mapping of antigenic sequence with truncated minigenes of E5 was necessary prior to epitope prediction.

The isolated TRAV17 and TRBV7-9 sequences resembled a CMV-specific TCR. TCR sequences specific for CMV pp65 and restricted to HLA-B*07:02 have been published with one to five amino acids difference in the CDR3 regions (66,67) (**Table 1**). These TCRs had been reported to recognize the TPRVTGGGAM (SEQ ID NO: 10) 10-mer epitope of CMV pp65 when presented on HLA-B*07:02. Here, pp65 TCR-transduced PBMCs were cocultured with K562-B*07:02 target cells loaded with CMV pp65-derived epitope p1 (TPRVTGGGAM, SEQ ID NO: 10) (**Fig. 12**a). Indeed, TCR-transduced T cells recognized p1-pulsed target cells and released IFNγ (**Fig. 12**b). pp65 TCR-transduced PBMCs were

specific for p1, despite sequence differences in the CDR3 region compared to previously published pp65-B*07:02-specific TCRs.

In summary, this approach enabled the identification of a novel immunogenic HPV16 E5 epitope and its corresponding TCR and of an immunodominant CMV pp65 epitope and its corresponding TCR. Both TCRs were specific for endogenously processed epitopes and reflected the T cell response caused by only one T cell clonotype in the initial MHC cell library-based screening. Thus, an unbiased screening of natural T cell responses and the identification of TCRs rapidly after antigen-specific *in vitro* stimulation is possible with the method of the invention, without prior knowledge of the epitope, thereby avoiding limitations of epitope prediction programs to predict functional T cell responses to a defined antigen and avoiding resource-intensive and unfavorable T cell clone culture. This approach can also be applied to TILs or tissue-resident T cells and screenings can be extended to further pathogen-derived and tumor-specific antigens as well as any antigen to be targeted by a TCR.

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Claims

1. A nucleic acid encoding a T cell receptor (TCR) alpha chain construct (TRA) and/or TCR beta chain construct (TRB) of a TCR construct specific for an epitope in complex with a human MHC I, wherein the epitope is an epitope of human papillomavirus 16 oncoprotein E5,

   wherein the nucleic acid is preferably obtainable from a method comprising
   (a) stimulating T cells isolated from a donor with professional antigen presenting cells presenting epitopes of said defined antigen and sharing at least one MHC allele with the donor, to enrich antigen-specific T cells; and
   (b) contacting said T cells with a library of cells, wherein each cell expresses a single MHC allele, wherein the library comprises cells expressing all MHC I alleles present in the donor, and wherein the cells of said library present epitopes of said defined antigen; and
   (c) selecting T cells activated by said contact, preferably, based on an activation marker expressed by said activated T cells; and
   (d) isolating the nucleic acids encoding the TCR alpha and TCR beta chains of the TCR of said T cells.

2. The nucleic acid of claim 1, wherein the epitope is an epitope of human papillomavirus 16 oncoprotein E5 restricted to HLA-B*15:01, which preferably comprises SEQ ID NO: 1.

3. The nucleic acid of claim 2, wherein the TRA comprises a CDR3 having at least 84% sequence identity to SEQ ID NO: 2, wherein the CDR3 preferably contains SEQ ID NO: 2, wherein the variable region of the TRA preferably has at least 80% sequence identity to SEQ ID NO: 3, and wherein the TRA preferably comprises SEQ ID NO: 4 and is encoded by SEQ ID NO: 5;
   and/or wherein the TRB comprises a CDR3 having at least 84% sequence identity to SEQ ID NO: 6, wherein the CDR3 preferably contains SEQ ID NO: 6, wherein the variable region of TRB preferably has at least 80% sequence identity to SEQ ID NO: 7, and wherein the TRA preferably comprises SEQ ID NO: 8 and is encoded by SEQ ID NO: 9.

4. A nucleic acid encoding a TRA and/or TRB of a TCR construct specific for an epitope in complex with a human MHC I, wherein the TCR construct is specific for an epitope of human cytomegalovirus protein pp65 in complex with HLA-B*07:02, which epitope consists of SEQ ID NO: 10, wherein the TRA comprises a CDR3 according to SEQ ID NO: 11 and/or wherein the TRB comprises a CDR3 according to SEQ ID NO: 15.

5. The nucleic acid of claim 4, wherein the variable region of the TRA has at least 80% sequence identity to SEQ ID NO: 12, and wherein the TRA preferably comprises SEQ ID NO: 13 and is

encoded by SEQ ID NO: 14; and/or wherein the variable region of the TRB has at least 80% sequence identity to SEQ ID NO: 16 and wherein the TRB preferably comprises SEQ ID NO: 17 and is encoded by SEQ ID NO: 18.

6. A host cell comprising a nucleic acid of any of the preceding claims encoding the TRA and TRB of a TCR construct specific for an epitope of human papillomavirus 16 oncoprotein E5 or for an epitope of human cytomegalovirus protein pp65, and expressing said TCR, wherein the host cell preferably is a CD8+ T cell.

7. A protein encoded by the nucleic acid of any of claims 1-5, preferably, comprising TRA and TRB.

8. The protein of claim 7, the recombinant T cell of claim 6, or the nucleic acid of any of claims 1-5, wherein the TCR construct comprises TRA and TRB, for use in a pharmaceutical composition, preferably, for treatment of a patient infected with
   a) human papillomavirus 16, wherein the TCR construct is specific for an epitope of human papillomavirus 16 oncoprotein E5 in complex with HLA-B*15:01 and wherein the patient is HLA-B*15:01-positive; or
   b) human cytomegalovirus, wherein the TCR construct is specific for an epitope of human cytomegalovirus protein pp65 in complex with HLA-B*07:02 and wherein the patient is HLA-B*07:02-positive.

9. A nucleic acid encoding a fragment of human papillomavirus 16 oncoprotein E5 having a length of up to 40 amino acids comprising an epitope, or a peptide fragment of human papillomavirus 16 oncoprotein E5 having a length of up to 40 amino acids comprising an epitope, wherein the epitope is capable of being recognized by the TCR construct of the T cell of claim 6,

   wherein the epitope is preferably identifiable by a method comprising
   (a) stimulating T cells isolated from a donor with professional antigen presenting cells presenting epitopes of said defined antigen and sharing at least one MHC allele with the donor, to enrich antigen-specific T cells; and
   (b) contacting said T cells with a library of cells, wherein each cell expresses a single MHC allele, wherein the library comprises cells expressing all MHC I alleles present in the donor, and wherein the cells of said library present epitopes of said defined antigen; and
   (c) selecting T cells activated by said contact, preferably, based on an activation marker expressed by said activated T cells; and
   (d) identifying the epitope capable of activating said selected T cells.

10. The nucleic acid or peptide of claim 9, wherein the E5 epitope comprises SEQ ID NO: 1 and is selected from the group consisting of SEQ ID NO: 1, 45, 47, 49, 51, 52 and 57.

11. The nucleic acid or peptide of any of claims 9 or 10 for use in a pharmaceutical composition, preferably, for preventing infection with human papillomavirus 16, or for treatment of a patient infected with human papillomavirus 16.

12. A method for preparing a nucleic acid encoding the TRA and TRB of a TCR construct specific for an epitope from a defined antigen presented on a MHC, comprising
    (a) stimulating T cells isolated from a donor with professional antigen presenting cells presenting epitopes of said defined antigen, to enrich antigen-specific T cells; and
    (b) contacting said T cells with a library of cells, wherein each cell expresses a single MHC allele, wherein the library comprises cells expressing all MHC I or MHC II alleles present in the donor, and wherein the cells of said library present epitopes of said defined antigen; and
    (c) selecting T cells activated by said contact, preferably, based on an activation marker expressed by said activated T cells; and
    (d) isolating the nucleic acids encoding the TCR alpha and TCR beta chains of the TCR of said T cells.

13. The method of claim 12, wherein the MHC is MHC I, wherein the library of cells preferably comprises MHC I-expressing K562 cells.

14. The method of any of claims 12 or 13, wherein the method further comprises optimizing the sequence, preferably, optimizing codon usage of the TRA and TRB and, optionally, combining human variable regions with murine constant regions or minimal murine constant regions.

15. A method for preparing a T cell expressing a TCR construct specific for an epitope from a defined antigen presented on a MHC, comprising carrying out the method of any of claims 12-14, and expressing said nucleic acids encoding the TRA and TRB in a T cell.

16. A method for identifying an epitope capable of being presented by a MHC in a defined antigen, comprising carrying out steps (a)-(d) of claim 12 and identifying the epitope capable of activating T cells transfected with nucleic acids encoding the isolated TRA and TRB constituting the TCR construct, wherein the epitope is optionally prepared in peptide or nucleic acid form.

Fig. 1

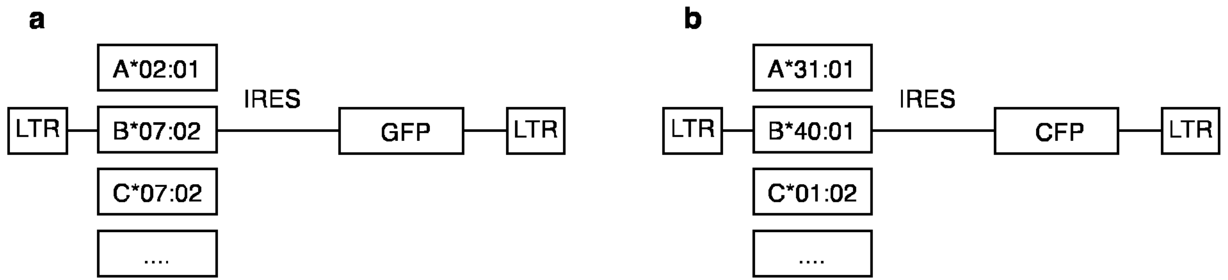


Fig. 2

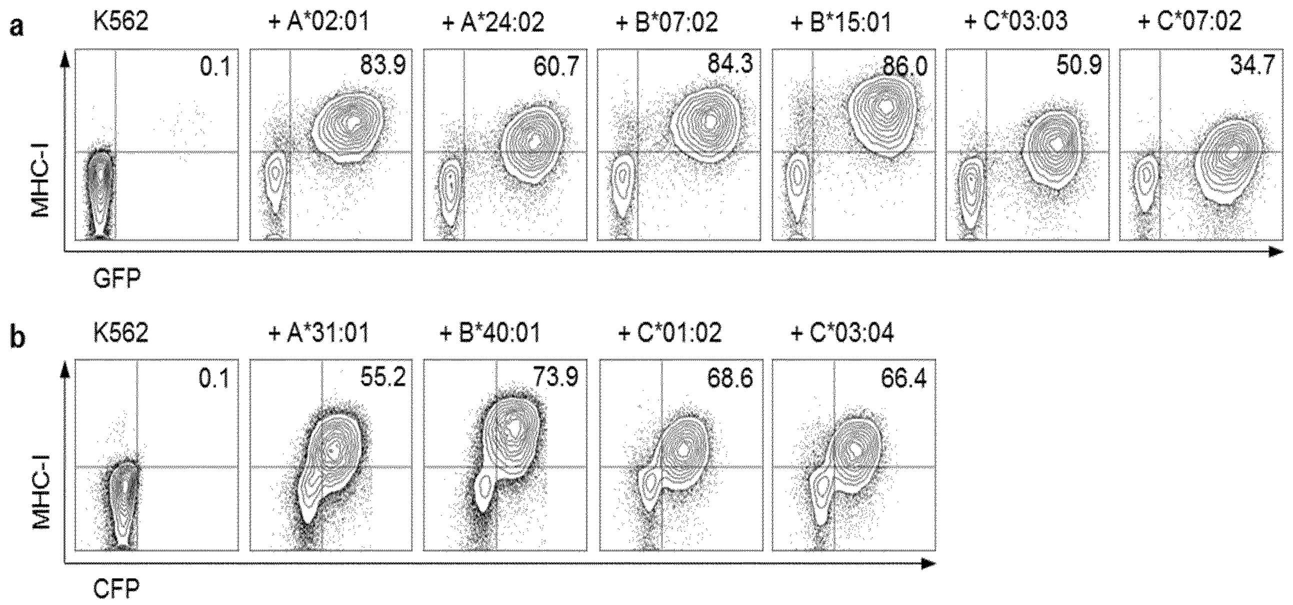


Fig. 3

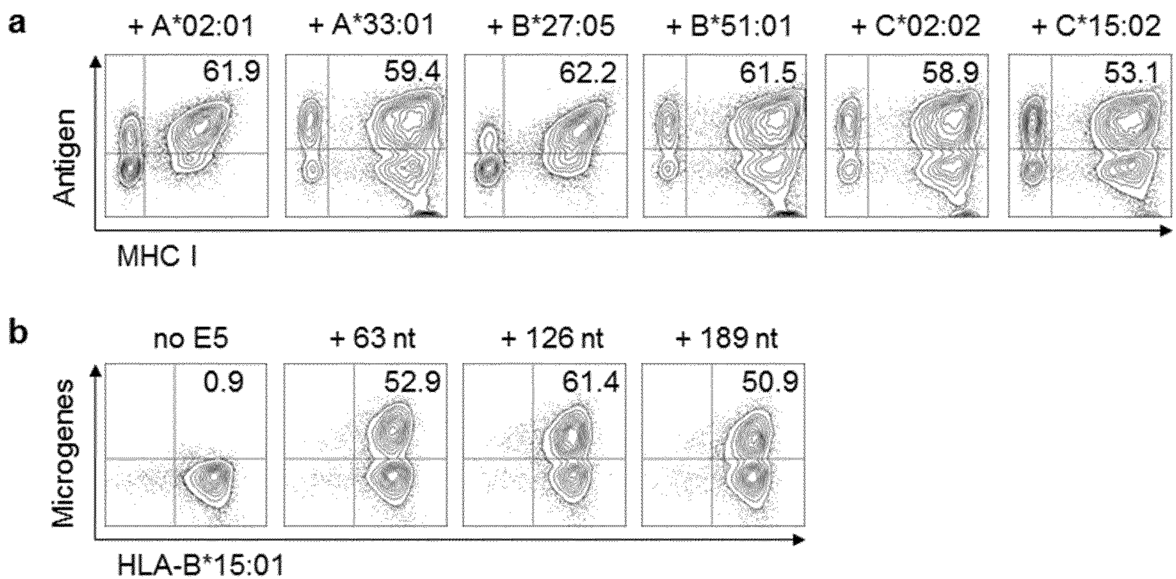


Fig. 4

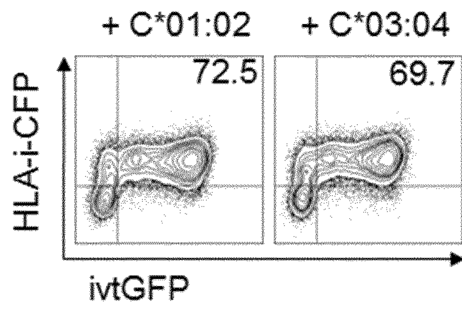


Fig. 5

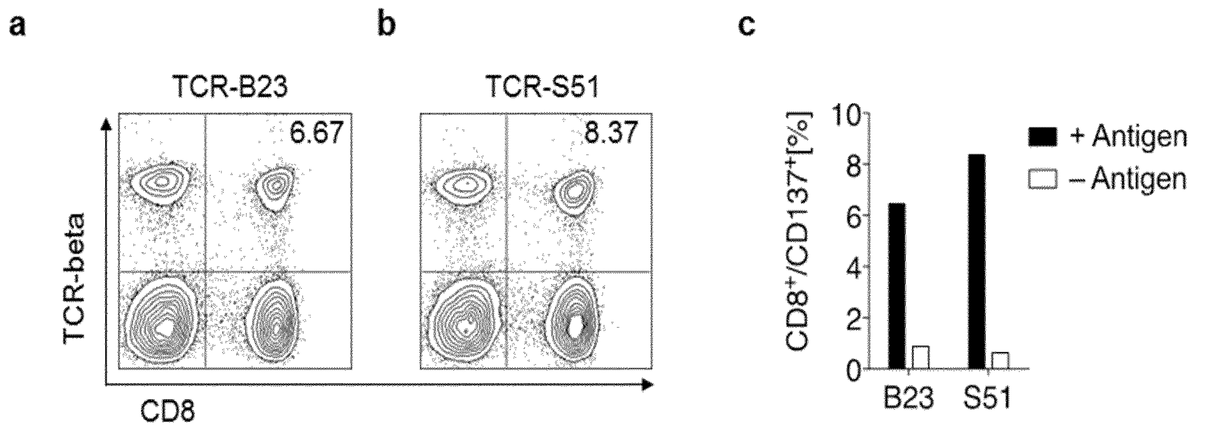


Fig. 6

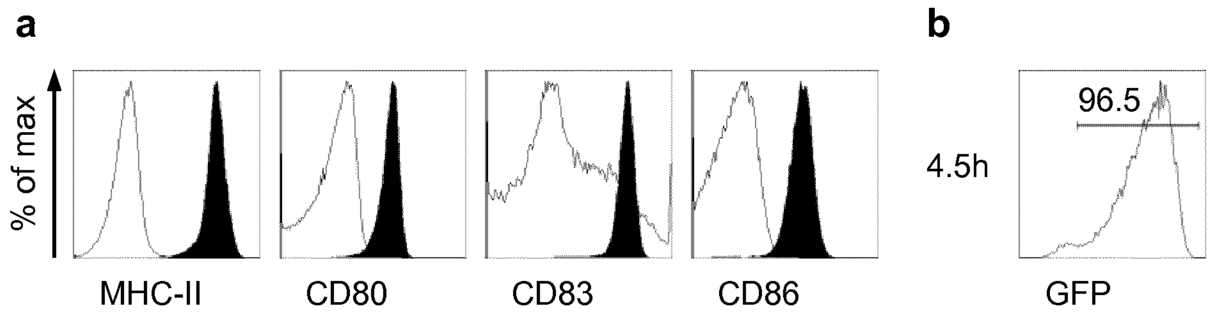


Fig. 7

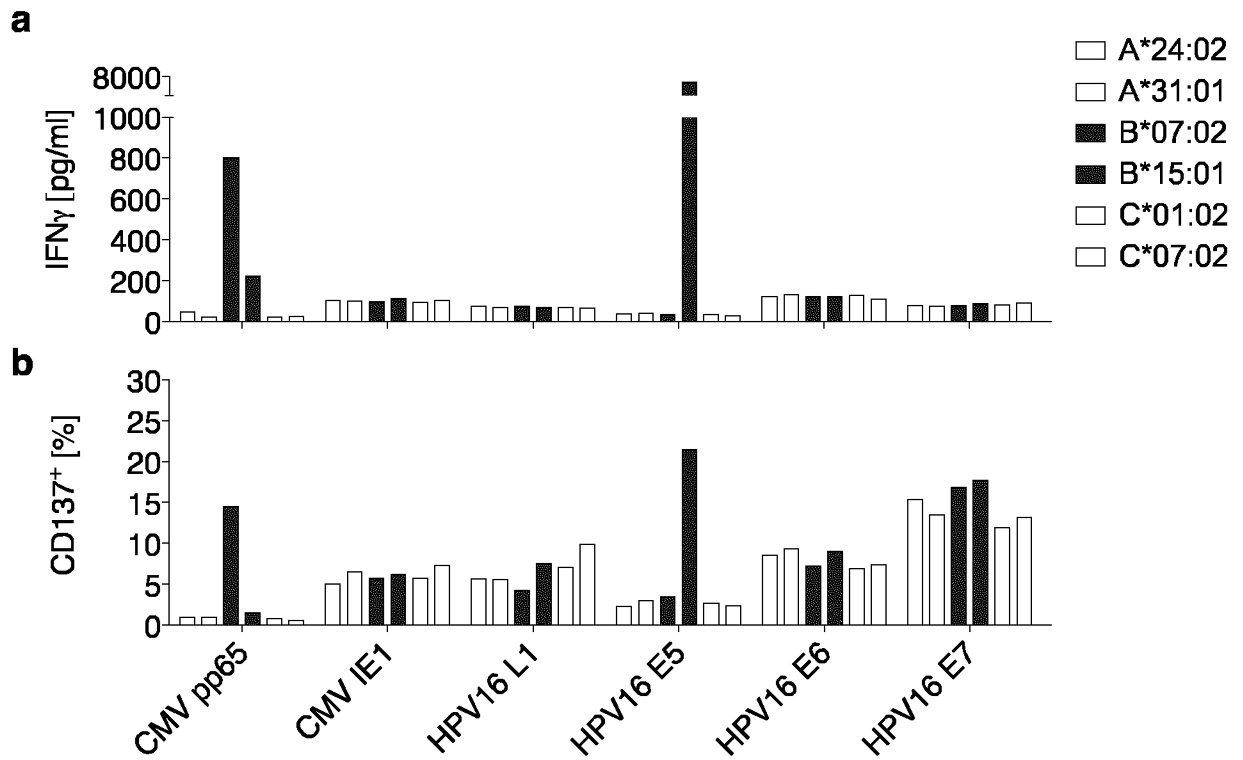


Fig. 8

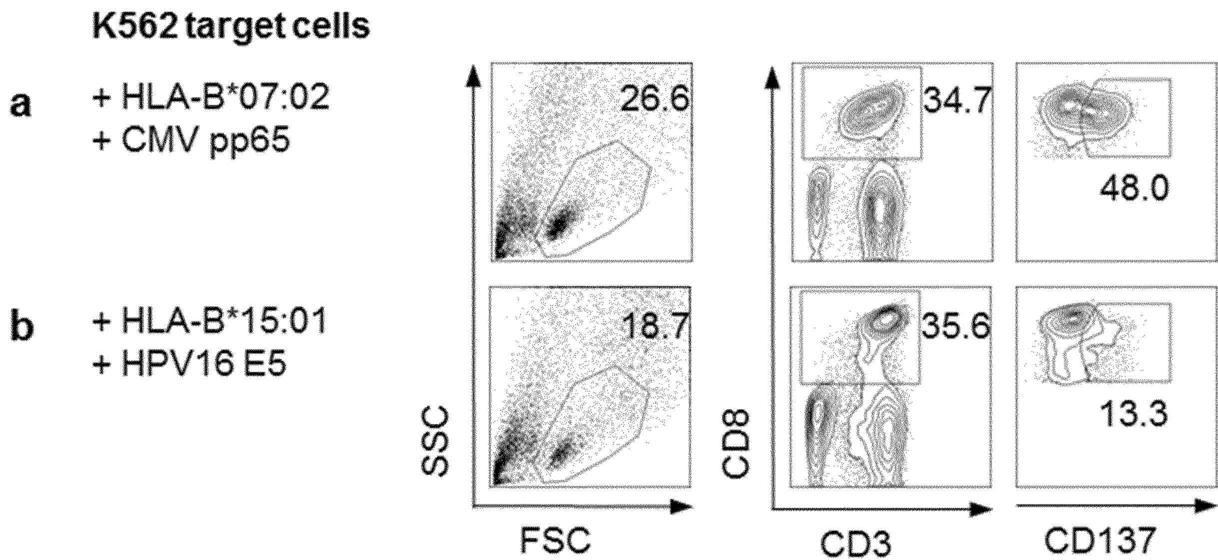
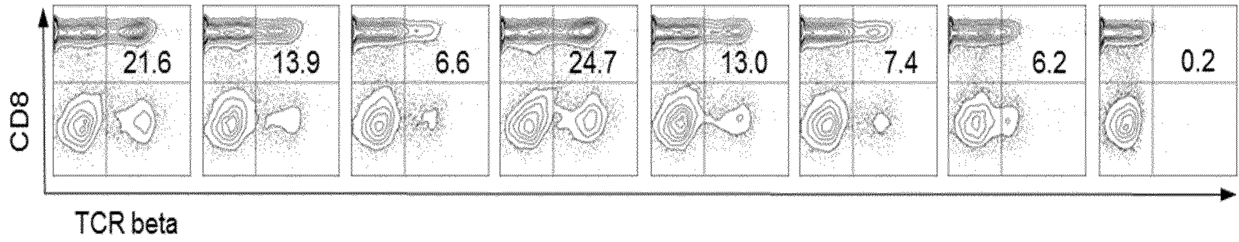


Fig. 9



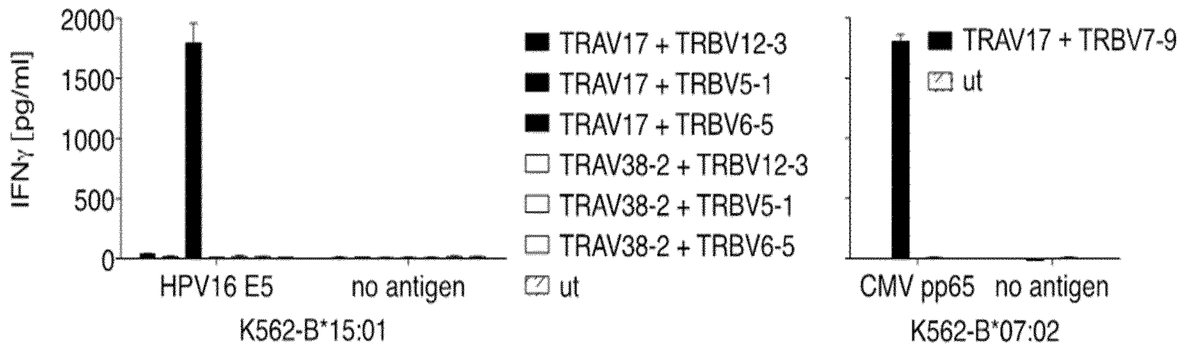


Fig. 10

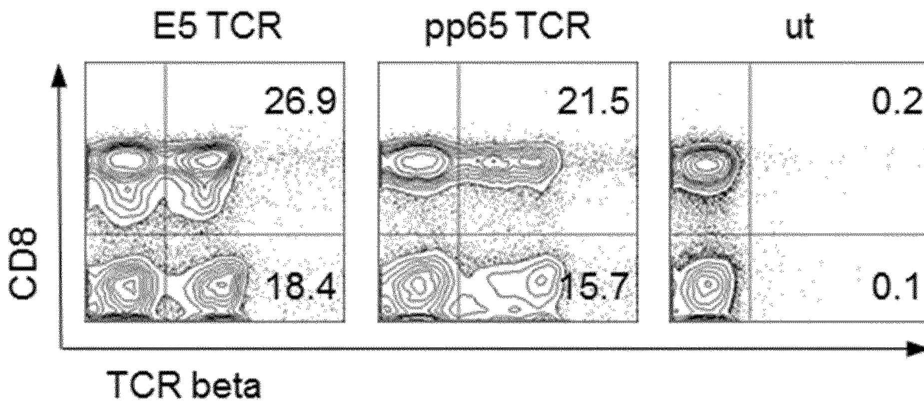


Fig. 11

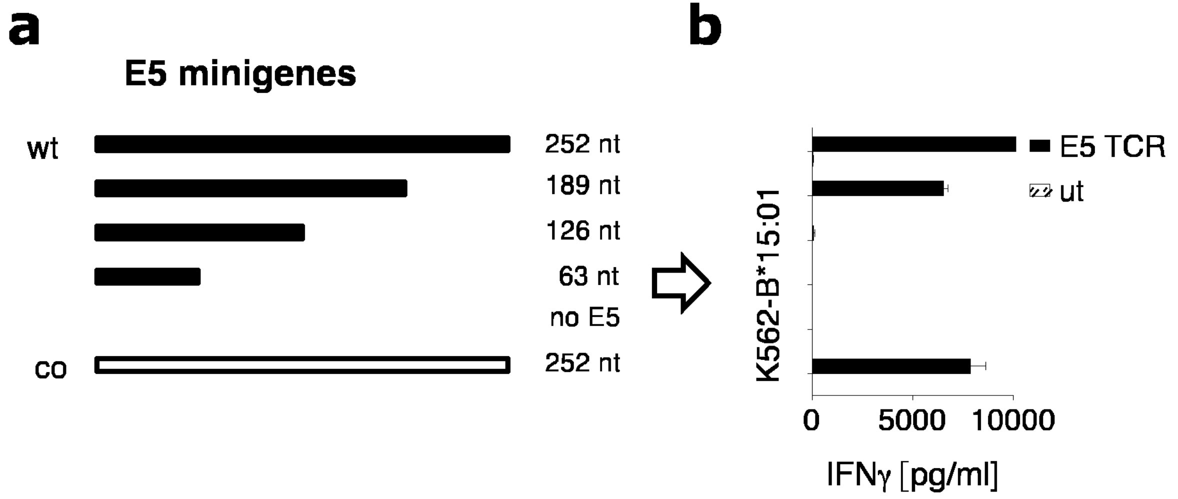


Fig. 12

a

HPV16 E5	-mer	Sequence
p4	9	SAFRCFIVY
p5	10	ASAFRCFIVY
p9	11	AASAFRCFIVY
p12	12	TAASAFRCFIVY
p7	13	ITAASAFRCFIVY
p11	14	WITAASAFRCFIVY
p15	8	WITAASAF
p6	10	LLWITAASAF
p13	11	LLLWITAASAF
p17	13	LLWITAASAFRCF
		no peptide

b

K562-B*15:01

E5 TCR

ut

0 4000 8000

IFNγ [pg/ml]

c

CMV pp65	-mer	Sequence
p1	10	TPRVTGGGAM
		no peptide

d

K562-B*07:02

pp65 TCR

ut

0 15000 30000

IFNγ [pg/ml]

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055518

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/705 C12N5/0783
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, BIOSIS, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 24 May 2012 (2012-05-24), "MHC class I/antigenic peptide to generate reversible multimer SEQ ID:154.", XP002744177, retrieved from EBI accession no. GSP:AZU81351 Database accession no. AZU81351 sequence & WO 2012/044999 A2 (LUDWIG INST FOR CANCER RES LTD [US]; LUESCHER IMMANUEL F [CH]; SCHMIDT) 5 April 2012 (2012-04-05) abstract example 2; table 1 sequence 154 ----- -/--	4-8

[X] Further documents are listed in the continuation of Box C.

[ ] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 May 2016	09/06/2016

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Voigt-Ritzer, Heike

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055518

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE Geneseq [Online] 6 May 2004 (2004-05-06), "Human papillomavirus peptide #770.", XP002744178, retrieved from EBI accession no. GSP:ADK08715 Database accession no. ADK08715 sequence & WO 2004/011650 A2 (INTERCELL AG [AT]; MATTNER FRANK [AT]; SCHMIDT WALTER [AT]; HABEL ANDR) 5 February 2004 (2004-02-05) table 7; sequence 770 -----	9-11
A	NICHOLAS P. RESTIFO ET AL: "Adoptive immunotherapy for cancer: harnessing the T cell response", NATURE REVIEWS IMMUNOLOGY, vol. 12, no. 4, 1 April 2012 (2012-04-01), pages 269-281, XP055034896, ISSN: 1474-1733, DOI: 10.1038/nri3191 the whole document figures 1,2 -----	1-16
X	CHEN Y-F ET AL: "Cytotoxic-T-lymphocyte human papillomavirus type 16 E5 peptide with CpG-oligodeoxynucleotide can eliminate tumor growth in C57BL/6 mice", JOURNAL OF VIROLOGY, THE AMERICAN SOCIETY FOR MICROBIOLOGY, US, vol. 78, no. 3, 1 February 2004 (2004-02-01), pages 1333-1343, XP003011807, ISSN: 0022-538X, DOI: 10.1128/JVI.78.3.1333-1343.2004 abstract figures 2,3 -----	1-3,5-8
A	ZENG WANYONG ET AL: "Artificial antigen-presenting cells expressing CD80, CD70, and 4-1BB ligand efficiently expand functional T cells specific to tumor-associated antigens", IMMUNOBIOLOGY, vol. 219, no. 8, 20 March 2014 (2014-03-20), pages 583-592, XP028868244, ISSN: 0171-2985, DOI: 10.1016/J.IMBIO.2014.03.003 cited in the application the whole document -----	1-16

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C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOUCHERMA R ET AL: "HLA-A*01:03, HLA-A*24:02, HLA-B*08:01, HLA-B*27:05, HLA-B*35:01, HLA-B*44:02, and HLA-C*07:01 Monochain Transgenic/H-2 Class I Null Mice: Novel Versatile Preclinical Models of Human T Cell Responses", THE JOURNAL OF IMMUNOLOGY, THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS, US, vol. 191, no. 2, 15 July 2013 (2013-07-15), pages 583-593, XP002744179, ISSN: 0022-1767, DOI: 10.4049/JIMMUNOL.1300483 [retrieved on 2013-06-17] cited in the application the whole document -----	1-16