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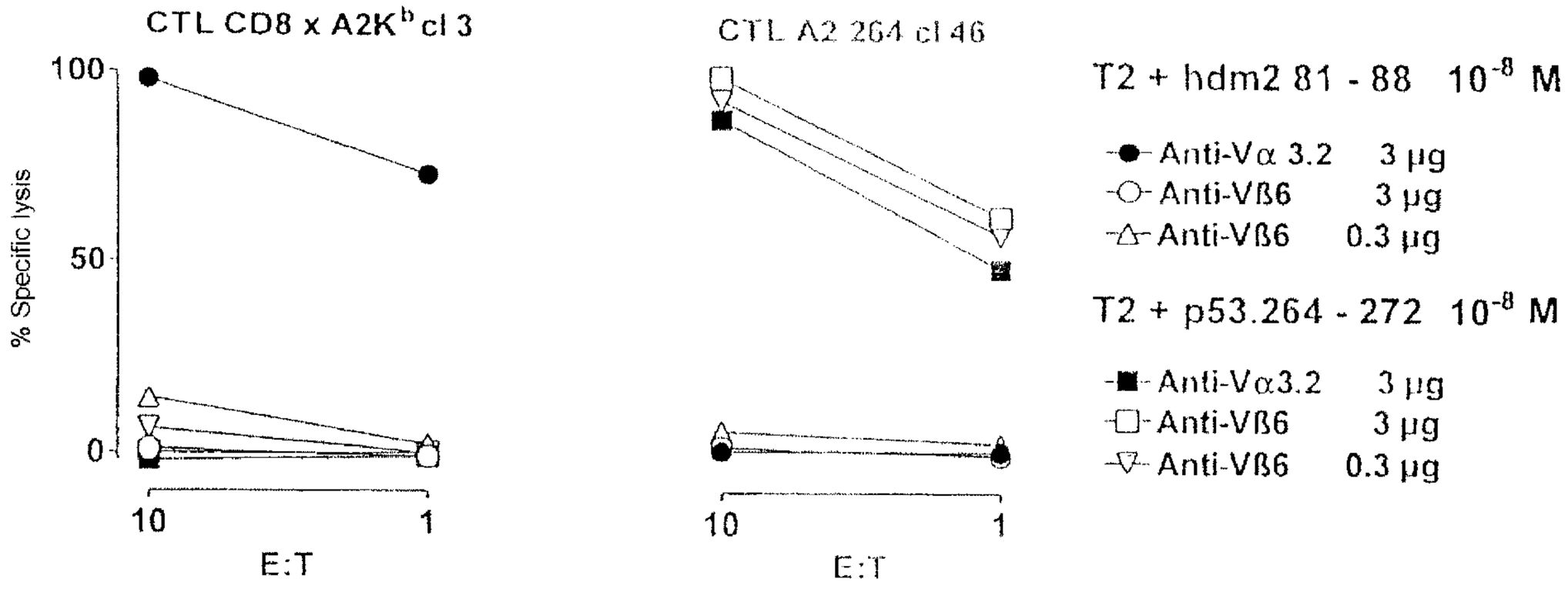
(54) Titre : POLYPEPTIDES D'UN RECEPTEUR DE LYMPHOCYTES T ALPHA/BETA DES MURIDES SPECIFIQUE DE LA PROTEINE HDM^{2}, ACIDES NUCLEIQUES LES CODANT ET LEUR UTILISATION

(54) Title: POLYPEPTIDES OF AN HDM^{2} PROTEIN-SPECIFIC MURINE ALPHA/BETA T-CELL RECEPTOR, NUCLEIC ACIDS ENCODING THESE AND THEIR USE



(57) Abrégé/Abstract:
The invention relates to polypeptides from a murine α/β T-cell receptor, mediating a hdm2 protein-specific T-cell response, or functional variants or parts thereof, or nucleic acids coding for the above, functional variants, or parts thereof. The effect of the above is that hdm2 protein-expressing cells are recognised by t-cells provided with said genes, cytokines are expressed and a t-cell-induced lysis and/or apoptosis of tumour- or leukaemic-cells is induced.

Abstract

The invention relates to polypeptides from a murine α/β T-cell receptor, mediating a hdm2 protein-specific T-cell response, or functional variants or parts thereof, or nucleic acids coding for the above, functional variants, or parts thereof. The effect of the above is that hdm2 protein-expressing cells are recognised by t-cells provided with said genes, cytokines are expressed and a t-cell-induced lysis and/or apoptosis of tumour- or leukaemic-cells is induced.

PCT/EP02/02187

Polypeptides of an hdm2 protein-specific murine α/β T-cell receptor, nucleic acids encoding these and their use

Description

The invention relates to polypeptides of the murine α/β T-cell receptor mediating an hdm2 protein-specific T-cell response, nucleic acids encoding these and their use in the therapy, diagnosis and/or prevention of diseases associated with the hdm2 protein.

Antigen recognition by T lymphocytes (TCL) is crucial for the generation and regulation of an effective immune response. The characteristic T-cell line marker is the T-cell antigen receptor (TCR). There are two defined types of TCR: One is a heterodimer of two disulfide-bonded polypeptides (α and β); the other is admittedly structurally similar, but consists of γ- and δ-polypeptides. Both receptors are associated with a set of five polypeptides, the CD3 complex, and thus together form the T-cell receptor complex (TCR-CD3 complex). The α/β-TCR is the most important functionally, since it is expressed in over 95% of all T-cells and mediates the principal immune response.

α/β T-cells can be subdivided into two different overlapping populations: A subgroup which carries the CD4 marker and mainly assists the immune response (T_H) and a subgroup which carries the CD8 marker and is essentially cytotoxic (T_C). $CD8^+$ T-cells recognize antigens in association with MHC class I molecules. Such antigens can, inter alia, be tumor-specific or tumor-associated peptide antigens. After recognition of the peptide antigens, the cell concerned is destroyed by the T-cell lyzing the target cell and/or inducing apoptosis of these target cells or releasing cytokines (e.g. IL-2, IFN-γ).

Among the tumor-associated peptide antigens (TAA), which in the context of MHC class I molecules are presented on the surface of tumor cells, the "universal" TAA are of particular interest. These TAA are derived mainly from cellular proteins, which are weakly expressed in normal cells and overexpressed in tumor cells. These proteins include, inter alia, the human homolog of the "mouse double minute 2" proto-antigen (mdm2), the "human mdm2" or in short "hdm2" proto-oncoprotein (Roth et al., 1998), which is over-expressed not only in a number of solid tumors, but also in the hematological neoplasias (malignant hematological systemic diseases) AML, ALL and CLL (Zhou et al, 2000).

Oligopeptides of the hdm2 protein can be presented to the cell-surface in context with MHC class I molecules and represent attractive target structures for CD8-positive T-cells. The extent of the T-cell response in this case proceeds in a defined kinetic window (Kersh et al., 1998). The complex of peptide MHC and TCR-CD3 multimerizes in order to bring about efficient signal transduction, the exact stoichiometry and the extent of the oligomerization still being disputed.

A prerequisite for the development of immuno-therapeutic procedures for the treatment of malignant oncoses is the identification of oncoprotein-specific T-cell receptors. Using such T-cell receptors, under certain conditions T-cells can be provided with antigen specificity in general and tumor reactivity in particular, with the aim that the T-cells bring about the remission and the eradication of a certain tumor. Described methods for the identification of high-affinity peptide-specific TCRs comprise the "phage" or "yeast-display" methodology (Holler et al., 1999) or a tetramer-dependent "TCR display" methodology (Kessels et al., 2000).

From Theobald et al. ("Targeting p53 as a general tumor antigen", P.N.A.S. USA 92, pp. 11993-11997, 1995), the preparation of p53-specific cytotoxic T-cells after the injection of peptides from the sequence of p53 is known. By means of these T-cell lines, it was subsequently possible to lyze a selection of human tumor cells. The isolation of genes of specific T-cell receptors of the lytic T-cells directed against p53 is not described. WO 97/32603 generally describes a process for the preparation of recombinant T lymphocytes, which express specific T-cell receptors directed against tumor tissue. In this process, an HLA-transgenic mouse (in this case HLA-A2) is immunized with tumor-associated antigen in order thus to bring about the production of cytotoxic T lymphocytes which express specific T-cell receptors on their surface. As tumor-associated antigens, peptides of various genes, such as Her-2/neu, Ras, p53, tyrosinase, MART, gp100, MAGE, BAGE and MUC-1 are described. From the Her-2/neu-specific T lymphocytes, the nucleotide sequence which contains at least one variable region of the α- and β-chain of the corresponding nonhuman T-cell receptor is isolated and used in the form of various genetic (inter alia "humanized") TCR constructs modified by molecular biology. Thus, WO 97/32603 describes fusion proteins of variable regions of TCR with the ζ-region of CD3 or CD8 or CD16, and also the use of flexible linkers of the amino acid sequence $(GGGGS)_3$.

Clay et al. ("Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity"; J. Immunology, 1999, 163: 507-513) describe the isolation of genes of the α-TCR and β-TCR chains of a MART-1 specific TCR and its expression in human peripheral blood lymphocytes (PBLs). The TCR are directed against the amino acids 25-35 of MART-1. Furthermore, the lysis of various metastatic melanoma cell lines and a retroviral vector system using IRES elements is described.

Darcy et al. ("Redirected perforin-dependent lysis of colon carcinoma by ex vivo genetically engineered CTL" J. Immunol., 2000, 164:3705-3712) describe an immunotherapeutic procedure for colon carcinoma using scFv anti-CEA receptor transduced CTL, perforin and γ-IFN. The chimeric specific receptor construct is transduced into primary mouse T-lymphocytes by means of a retroviral vector. These cells were injected into mice which had previously been inoculated with colon carcinoma cell lines.

Weijtens et al. ("A retroviral vector system ,'STITCH'; in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T-lymphocytes", Gene Therapy (1998) 5, 1995-1203) and Willemsen et al. "Grafting primary human T lymphocytes with cancer-specific single chain and two chain TCR", Gene Therapy (2000) 7, 1369-1377) describe retroviral vector systems for the transduction of genes into activated T-lymphocytes. This system is used in order to bring about the expression of antibody-based chimeric receptors in the membrane of T-cells. These T-cells can then be employed against specific cancer cells. The activity of the retroviral vector system is described by means of the specific recognition and cytolysis of renal cell carcinomas. Similarly, Eshar. et al. ("Specific activation and targeting of cytotoxic lymphocytes through chimeric single chains consisting of antibody-binding domains and the γ or ζ subunits of the immunoglobulin and T-cell receptors", P.N.A.S. USA, 90, pp. 720-724, 1993) describe the preparation of tumor-specific lymphocytes and their use in immunotherapy on the basis of chimeras, which comprise the variable regions of an antibody (scFV's) with the constant region of the T-cell receptor ("single-chain TCR" (scTCR)). It was possible for these chimeric genes to be expressed on the surface of cytolytic T-cell

hybridomas and they brought about the secretion of interleukin-2 after contact with the antigen.

A TCR directed specifically against hdm2 and its use is, however, neither mentioned nor suggested by the abovementioned publications and in the other literature.

The present invention is therefore based on the object of making available the murine genes of the T-cell receptors α-TCR and β-TCR directed against the hdm2 protein. These cause hdm2 protein-expressing cells of CD8-positive T-cells to be recognized, cytokines to be secreted, and a T-cell-induced lysis and/or apoptosis of tumor or leukemia cells to be produced.

According to the invention, this object is achieved by the polypeptide of the murine α/β T-cell receptor mediating an hdm2 protein-specific T-cell response according to SEQ ID No. 1 or SEQ ID No. 2 or functional variants or parts thereof or nucleic acids encoding this, functional variants or parts thereof.

From a double-A2.1 transgenic mouse [huCD8α/β x $A2.1/K^b]_{F1}$ in C57BL/6 genetic background, the genes of the murine α/β T-cell receptor mediating an hdm2 protein-specific T-cell response were isolated, which mediate a specific T-cell response which is HLA-A2.1-restricted and directed against the peptide of the amino acids 81-88 of the hdm2 protein. The polypeptides of this TCR were hitherto unknown. The genes were inserted retrovirally into human peripheral blood lymphocytes (PBLs) as wild-type (WT) or modified constructs and the HLA-restricted antigen recognition was checked functionally by means of CD8-mediated cytotoxic lysis of various cell lines in the ^{51}chromium-release test. The genes of the hdm2-specific TCR according to the invention are not part of the previously known suitable targets for the diagnosis -

such as, for example, the indication - and/or the treatment - such as, for example, the modulation - of diseases connected with hdm2 protein or for the identification of pharmacologically active substances, such that completely novel therapeutic approaches result from this invention.

A further aspect of the invention relates to a fusion protein, comprising the polypeptide according to the invention or functional variants or parts thereof or nucleic acids encoding this, functional variants or parts thereof.

The fusion protein can be characterized in that it comprises the ζ-region von CD3 oder CD8 or CD16 or parts thereof, in particular the ζ-region of human CD3 or CD8 or CD16 or parts thereof. A fusion protein according to the invention is preferred which comprises a flexible linker (Whitlow et al., "An improved linker for single-chain Fv with reduced aggregation and enhanced proteolytic stability", Prot. Engin. 6(8), pp. 989-995, 1993), in particular a linker of the amino acid sequence $(GGGGS)_3$. In particular, the fusion protein according to the invention can comprise the ζ-chain of the CD3-complex or ITAM motifs of the ζ-chain or parts thereof, in particular the ζ-chain of human CD3 or parts thereof. The fusion protein can furthermore be characterized in that it comprises CD8α or the Lck-binding motif of CD8α or parts thereof, in particular of human CD8α.

The fusion protein according to the invention can furthermore be a chimeric partially or completely humanized α and/or β TCR chain, in particular according to SEQ ID No. 3 or SEQ ID No. 4. A further aspect of the invention relates to a fusion protein which is a single-chain T-cell receptor, in particular according to SEQ ID No. 5 or SEQ ID No. 6. The fusion protein according to the invention can, however, also be

characterized in that it is an α/β-TCR coupled to other functional components, in particular according to SEQ ID No. 7 or SEQ ID No. 8.

A further subject of the invention is a process for the preparation of a fusion protein according to the invention for the diagnosis and/or treatment of diseases connected with hdm2 protein or for the identification of pharmacologically active substances, e.g. in a suitable host cell, in which a nucleic acid according to the invention is used.

Fusion proteins are prepared here which contain the polypeptides according to the invention described above, where the fusion proteins themselves already have the function of a polypeptide of the invention or the specific function is active only after removal of the fusion portion. These especially include fusion proteins having a proportion of about 1-200, preferably about 1-150, in particular about 1-100, especially about 1-50 foreign amino acids. Examples of such peptide sequences are prokaryotic peptide sequences, which can be derived, for example, from the galactosidase of *E. coli*. Furthermore, viral peptide sequences, such as, for example, of the bacteriophage M13, can also be used in order thus to produce fusion proteins for the "phage display" process known to the person skilled in the art.

For the purification of the proteins according to the invention, a further polypeptide ("tag") can be added. Protein tags according to the invention allow, for example, high-affinity absorption on a matrix, stringent washing with suitable buffers without eluting the complex to a noticeable extent, and subsequently the elution of the absorbed complex in a controlled manner. Examples of the protein tags known to the person skilled in the art are a $(His)_6$ tag, a Myc tag, a FLAG tag, a Strep tag, a Strep tag II, a

hemagglutinin tag, glutathione transferase (GST) tag, intein with an affinity chitin-binding tag or maltose-binding protein (MBP) tag. These protein tags can be located N-, C-terminally and/or internally.

Beside the natural polypeptides isolated from cells, all polypeptides according to the invention or their parts can have been prepared under cell-free conditions, e.g. by synthesis or by *in vitro* translation. Thus, the entire polypeptide or parts thereof can be synthesized, for example, with the aid of the classical synthesis (Merrifield technique). Parts of the polypeptides according to the invention are suitable, in particular, for the obtainment of antisera, with the aid of which suitable gene expression banks can be searched in order thus to obtain further functional variants of the polypeptide according to the invention.

The invention also relates to polypeptides which are derivatives of an antibody having a specificity for hdm2-81-88 peptide, in particular presented in the context of HLA-A2.1.

The invention furthermore comprises retro-inverse peptides or pseudopeptides according to the polypeptide sequence of SEQ ID No. 1 to SEQ ID No. 8 or functional variants or parts thereof. Instead of the -CO-NH- peptide bonds, these peptides have -NH-CO- bonds.

The object of the invention is furthermore achieved by a nucleic acid according to the invention, which is a DNA, RNA, PNA (peptide nucleic acid) or p-NA (pyranosyl nucleic acid), preferably a DNA, in particular is a double-stranded DNA having a length of at least 8 nucleotides, preferably having at least 18 nucleotides, in particular having at least 24 nucleotides. The nucleic acid can be characterized in that the sequence of the nucleic acid has at least one intron and/or one

polyA sequence. It can also be present in the form of its antisense sequence.

For the expression of the gene concerned, in general a double-stranded DNA is preferred, the DNA region coding for the polypeptide being particularly preferred. This region begins with the first start codon (ATG) in a Kozak consensus sequence (Kozak, 1987, Nucleic Acids Res. 15:8125-48) up to the next stop codon (TAG, TGA bzw. TAA), which is in the same reading frame with respect to the ATG. A further use of the nucleic acid sequences according to the invention is the construction of anti-sense oligonucleotides (Zheng and Kemeny, 1995, Clin. Exp. Immunol. 100:380-2) and/or ribozymes (Amarzguioui et al. 1998, Cell. Mol. Life Sci. 54:1175-202; Vaish et al., 1998, Nucleic Acids Res. 26:5237-42; Persidis, 1997, Nat. Biotechnol. 15:921-2). Using anti-sense oligonucleotides, it is possible to decrease the stability of the nucleic acid according to the invention and/or to inhibit the translation of the nucleic acid according to the invention. Thus it is possible, for example, to decrease the expression of the corresponding genes in cells both in vivo and in vitro. Oligonucleotides can therefore be suitable as a therapeutic. This strategy is suitable, for example, also for the skin, epidermal and dermal cells, in particular if the antisense oligonucleotides are complexed with liposomes (Smyth et al., 1997, J. Invest. Dermatol. 108:523-6; White et al., 1999, J. Invest. Dermatol. 112:699-705). For use as a probe or as an "antisense" oligonucleotide, a single-stranded DNA or RNA is preferred.

Beside the natural nucleic acids isolated from cells, all nucleic acids according to the invention or their parts can also have been prepared synthetically. Furthermore, for carrying out the invention, a nucleic acid can be used which has been prepared synthetically. Thus, the nucleic acid according to the invention can

be synthesized, for example, chemically with the aid of the protein sequences described in SEQ ID No. 1 to SEQ ID No. 8 making use of the genetic code, e.g. according to the phosphotriester method (see e.g. Uhlmann, E. & Peyman, A. (1990) Chemical Reviews, 90, 543-584).

Oligonucleotides are as a rule degraded rapidly by endo- or exonucleases, in particular by DNases and RNases occurring in the cell. It is therefore advantageous to modify the nucleic acid in order to stabilize it against degradation, so that a high concentration of the nucleic acid is maintained in the cell over a long period of time (WO 95/11910; Macadam et al, 1998, WO 98/37240; Reese et al, 1997, WO 97/29116). Typically, such a stabilization can be obtained by the introduction of one or more internucleotide phosphorus groups or by the introduction of one or more non phosphorus internucleotides.

Suitable modified internucleotides are summarized in Uhlmann and Peymann (1990 Chem. Rev. 90, 544) (WO 95/11910; Macadam et al., 1998, WO 98/37240; Reese et al., 1997, WO 97/29116). Modified internucleotide phosphate radicals and/or non phosphorus bridges in a nucleic acid which can be employed in one of the uses according to the invention, contain, for example, methylphosphonate, phosphorothioate, phosphor-amidate, phosphorodithioate, phosphate esters, while non phosphorus internucleotide analogs, for example, contain siloxane bridges, carbonate bridges, carboxy-methyl esters, acetamidate bridges and/or thioether bridges. It is also intended that this modification improves the stability of a pharmaceutical composition which can be employed in one of the uses according to the invention.

A further aspect of the present invention relates to a vector, preferably in the form of a plasmid, shuttle vector, phagemid, cosmid, expression vector, adenoviral

vector, retroviral vector (Miller et al. "Improved retroviral vectors for gene transfer and expression", BioTechniques Vol. 7, No. 9, p 980, 1989) and/or a vector having gene therapy activity, which contains a nucleic acid according to the invention.

Thus the nucleic acid according to the invention can be contained in a vector, preferably in an expression vector or vector active in gene therapy. Preferably, the vector active in gene therapy contains T-cell-specific regulatory sequences, which are bound functionally to the nucleic acid according to the invention. The expression vectors can be prokaryotic or eukaryotic expression vectors. Examples of prokaryotic expression vectors are, for expression in *E. coli*, for example, the vectors pGEM or pUC derivatives and for eukaryotic expression vectors for expression in *Saccharomyces cerevisiae*, for example, the vectors p426Met25 or p126GAL1 (Mumberg et al. (1994) Nucl. Acids Res. 22, 5767-5768), for expression in insect cells, for example, *Baculovirus* vectors such as disclosed in EP-B1-0 127 839 or EP-B1-0 549 721, and for expression in mammalian cells, for example, the vectors Rc/CMV and Rc/RSV or SV40 vectors, which are all generally obtainable.

In general, the expression vectors also contain promoters suitable for the respective host cell, such as, for example, the trp promoter for expression in E. coli (see, for example, EP-B1-0 154 133), the Met 25, GAL 1 or ADH2 promoter for expression in yeasts (Russel et al. (1983), J. Biol. Chem. 258, 2674-2682; Mumberg, supra), the Baculovirus polyhedrin promoter for expression in insect cells (see e.g. 13. EP-B1-0 127 839). For expression in mammalian cells, suitable promoters are, for example, those which allow a constitutive, regulatable, tissue-specific, cell cycle-specific or metabolically specific expression in eukaryotic cells. Regulatable elements according to the

present invention are promoters, activator sequences, enhancer, silencer and/or repressor sequences. Examples of suitable regulatable elements which make possible constitutive expression in eukaryotes are promoters which are recognized by the RNA polymerase III or viral promoters, CMV enhancer, CMV promoter, CMV-LTR hybrids, SV40 promoter or LTR promoters, for example of MMTV (mouse mammary tumour virus; Lee et al. (1981) Nature 214, 228-232) and further viral promoter and activator sequences, derived from, for example, HBV, HCV, HSV, HPV, EBV, HTLV or HIV. An example of a regulatable element which makes possible regulatable expression in the eukaryotes is the tetracycline operator in combination with an appropriate repressor (Gossen M. et al. (1994) Curr. Opin. Biotechnol. 5,516-20).

Examples of regulatable elements which make possible T-cell-specific expression in eukaryotes are promoters or activator sequences of promoters or enhancers of those genes which code for proteins which are only expressed in these cell types.

Examples of regulatable elements which make possible cell cycle-specific expression in eukaryotes are promoters of the following genes: cdc25, cyclin A, cyclin E, cdc2, E2F, B-myb or DHFR (Zwicker J. and Müller R. (1997) Trends Genet. 13, 3-6). Examples of regulatable elements which make possible metabolically specific expression in eukaryotes are promoters which are regulated by hypoxia, by glucose deficiency, by phosphate concentration or by heat shock.

The vector according to the invention can be used for the transfection of a host cell which is preferentially a T-cell. Particularly preferred is a host cell which is characterized in that it expresses a polypeptide or fusion protein according to the invention on its surface.

In order to make possible the introduction of the nucleic acids according to the invention and thus the expression of the polypeptide in a eukaryotic or prokaryotic cell by transfection, transduction, transformation or infection, the nucleic acid can be present as a plasmid, as part of a viral or nonviral vector or particle. Suitable viral vectors or particles in this case are particularly: baculoviruses, vaccinia viruses, retroviruses, adenoviruses, adeno-associated viruses and herpesviruses. Suitable nonviral carriers in this case are particularly: virosomes, liposomes, cationic lipids, or poly-lysine-conjugated DNA.

Examples of vectors active in gene therapy are virus vectors, for example adenovirus vectors or retroviral vectors (Lindemann et al., 1997, Mol. Med. 3: 466-76; Springer et al., 1998, Mol. Cell. 2: 549-58; Weijtens et al. "A retroviral vector system, 'STITCH'; in combination with an optimized single chain antibody chimeric receptor gene structure allows efficient gene transduction and expression in human T-lymphocytes", Gene Therapy (1998) 5, 1935-1203).

A preferred mechanism for expressing polypeptides according to the invention *in vivo* is viral gene transfer, in particular with the aid of retroviral particles. These are preferably utilized to provide appropriate target cells, preferably T-lymphocytes, of the patient *ex vivo* with the genes or nucleotide sequences coding for polypeptides according to the invention by transduction. The target cells can afterwards be reinfused into the patient again in the sense of an adoptive cell transfer in order to take over tumoricidal and/or immunomodulating effector functions using the *de novo* inserted specificity. Recently, in this way very good gene therapy results were achieved in the treatment of the SCID-X1 disease characterized by immunoincompetence in newborn children in whom hematological precursor cells were provided

retrovirally with an analogous intact transgene of a nonfunctional mutated variant of the γ-chain gene occurring in the children, which is essential for the differentiation into the various effector cells of the adaptive immune system (Cavazzana-Calvo et al., 2000).

There is furthermore the possibility of carrying out the gene transfer *in vivo*, on the one hand by preferentially stereotactic injection of the infectious particles, on the other hand by direct administration of virus-producing cells (Oldfield et al., Hum. Gen. Ther., 1993, 4:39-69).

The viral vectors often employed for the transfer of genes are according to the present state of the art mainly retroviral, lentiviral, adenoviral and adeno-associated viral vectors. These are cyclic nucleotide sequences derived from natural viruses, in which at least the viral structural protein-coding genes are replaced by the construct to be transferred.

Retroviral vector systems create the prerequisite for a long-lasting expression of the transgene by the stable, but undirected integration into the host genome. Vectors of the younger generation possess no irrelevant and potentially immunogenic proteins, in addition there is no preexisting immunity of the recipient to the vector. Retroviruses contain an RNA genome which is packaged in a lipid coat which consists of parts of the host cell membrane and of virus proteins. For the expression of viral genes, the RNA genome is reverse-transcribed and integrated into the target cell DNA with the enzyme integrase. This can afterwards be transcribed and translated by the infected cell, whereby viral constituents result which combine to give retroviruses. RNA is exclusively then inserted into the newly formed viruses. The genome of the retroviruses possesses three essential genes: *gag*, which codes for viral structural proteins, "group-specific antigens",

pol for enzymes such as reverse transcriptase and integrase and *env* for the coat protein ("envelope"), which is responsible for the binding of the host-specific receptor. The production of the replication-incompetent viruses takes place after transfection in "packaging cell lines", which have additionally been equipped with the gag/pol-coding genes and express these "in trans" and thus complement the formation of replication-incompetent (i.e. gag/pol-deleted) transgenic virus particles. An alternative is the co-transfection of the essential virus genes, only the vector containing the transgene carrying the packaging signal.

The separation of these genes on the one hand makes possible the arbitrary combination of the gal/pol-reading frame with *env* reading frames obtained from various strains, whereby pseudotypes having modified host tropism result, on the other hand the formation of replication-competent viruses within packaging cells can be drastically reduced thereby. The coat protein derived from "gibbon ape leukemia virus" (GALV), which is used in the "stitch" or "bullet" vector system, is able to transduce human cells and is established in the packaging cell line PG13 with an amphotrophic host range (Miller et al., 1991). Additionally, the safety is increased by selective deletion of nonessential virus sequences for the prevention of a homologous recombination and thus the production of replication-competent particles.

Novel, nonviral vectors consist of autonomous, self-integrating DNA sequences, the transposons, which are inserted into the host cell by, for example, liposomal transfection and have for the first time been employed successfully for the expression of human transgenes in mammalian cells (Yant et al., 2000).

Vectors active in gene therapy can also be obtained by complexing the nucleic acid according to the invention with liposomes, since thereby a very high transfection efficiency, in particular of skin cells, can be achieved (Alexander and Akhurst, 1995, Hum. Mol. Genet. 4: 2279-85). Excipients which increase the transfer of nucleic acids to the cells can, for example, be proteins or peptides which are bound to DNA or synthetic peptide-DNA molecules which make possible the transport of the nucleic acid into the nucleus of the cell (Schwartz et al. (1999) Gene Therapy 6, 282; Brandén et al. (1999) Nature Biotech. 17, 784). Excipients also include molecules which make possible the release of nucleic acids into the cytoplasm of the cell (Planck et al. (1994) J. Biol. Chem. 269, 12918; Kichler et al. (1997) Bioconj. Chem. 8, 213) or, for example, liposomes (Uhlmann and Peymann (1990) supra). Another particularly suitable form of gene therapy vectors can be obtained by applying the nucleic acid according to the invention to gold particles and shooting these into tissue, preferably into the skin, or cells with the aid of the "gene gun" (Wang et al., 1999, J. Invest. Dermatol., 112:775-81).

For the gene therapy application of the nucleic acid according to the invention, it is also advantageous if the part of the nucleic acid which codes for the polypeptides contains one or more noncoding sequences including intron sequences, preferably between the promoter and start codon of the polypeptide, and/or a polyA sequence, in particular the naturally occurring polyA sequence or an SV40 virus polyA sequence, especially at the 3'-end of the gene, since by this means a stabilization of the mRNA can be achieved (Jackson, R. J. (1993) Cell 74, 9-14 and Palmiter, R. D. et al. (1991) Proc. Natl. Acad. Sci. USA 88, 478-482).

A further subject of the present invention is a host cell, in particular a T-cell, which is transformed using a vector according to the invention or another gene construct according to the invention. Host cells can be either prokaryotic or eukaryotic cells, examples of prokaryotic host cells are *E. coli* and of eukaryotic cells are *Saccharomyces cerevisiae* or insect cells.

A particularly preferred transformed host cell is a transgenic T-precursor cell or a stem cell, which is characterized in that it comprises a gene construct according to the invention or an expression cassette according to the invention. Processes for the transformation or transduction of host cells and/or stem cells are well known to the person skilled in the art and include, for example, electroporation or microinjection. A particularly preferred transformed host cell is a patient's own T-cell, which after removal is transfected using a gene construct according to the invention. Host cells according to the invention can in particular be obtained by removing from the patient one or more cells, preferentially T-cells, in particular $CD8^+$-T-cells, which are then transfected or transduced *ex vivo* using one or more genetic constructs according to the invention in order thus to obtain host cells according to the invention. The specific T-cells generated *ex vivo* can then subsequently be reimplanted into the patient. The process is thus similar to the process described in Darcy et al. ("Redirected perforin-dependent lysis of colon carcinoma by ex vivo genetically engineered CTL" J. Immunol., 2000, 164:3705-3712) using CTL transduced by scFv anti-CEA receptor, perforin and γ-IFN.

A further aspect of the invention relates to a process for the identification of hdm2 protein-specific antigens which is characterized in that hdm2-presenting tumor cells or fractions thereof are combined with a host cell according to the invention under conditions

in which the tumor cells or fractions thereof are only lyzed if the tumor presents hdm2 protein-specific antigen for which the expressed polypeptide or fusion protein is specific.

A further aspect of the invention relates to a process for the preparation of an antibody, preferably of a polyclonal or monoclonal antibody, for the diagnosis and monitoring of diseases associated with hdm2 protein or for the identification of pharmacologically active substances in which an antibody-producing organism is immunized with a polypeptide according to the invention or functional equivalents thereof or parts thereof having at least 6 amino acids, preferably having at least 8 amino acids, in particular having at least 12 amino acids or is vaccinated with a nucleic acid encoding a polypeptide according to the invention.

The process is carried out according to methods generally known to the person skilled in the art by immunizing a mammal, for example a rabbit, with the polypeptide according to the invention or the parts thereof mentioned, if appropriate in the presence of, for example, incomplete Freund's adjuvant and/or aluminum hydroxide gels (see, for example, Diamond, B. A. et al. (1981) The New England Journal of Medicine, 1344-1349). The polyclonal antibodies formed in the animal on account of an immunological reaction can then be easily isolated from the blood according to generally known methods and purified, for example, by means of column chromatography. Monoclonal antibodies can be prepared, for example, according to the known method of Winter & Milstein (Winter, G. & Milstein, C. (1991) Nature, 349, 293-299).

A further subject of the present invention is an antibody for the diagnosis and/or treatment of diseases associated with hdm2 protein or for the identification of pharmacologically active substances, which is

directed against a polypeptide according to the invention and specifically reacts with the polypeptides according to the invention, where the abovementioned parts of the polypeptide are either themselves immunogenic or can be increased in their immunogenicity by coupling to suitable carriers, such as, for example, bovine serum albumin. This antibody is either polyclonal or monoclonal, a monoclonal antibody is preferred. The term antibody is understood according to the present invention as also meaning prepared by genetic engineering and optionally modified antibodies or antigen-binding parts thereof, such as, for example, chimeric antibodies, humanized antibodies, multi-functional antibodies, monovalent or oligovalent antibodies, bi- or oligospecific antibodies, single-stranded antibodies, F(ab) or $F(ab)_2$ fragments (see, for example, EP-B1-0 368 684, US 4,816,567, US 4,816,397, WO 88/01649, WO 93/06213, WO 98/24884). The antibodies according to the invention can be used for the diagnosis and/or treatment of diseases associated with hdm2 protein or for the identification of pharmacologically active substances.

The present invention furthermore relates to a process for the production of a medicament for the treatment of diseases associated with hdm2 protein, characterized in that at least one nucleic acid, at least one polypeptide, at least one host cell or at least one antibody as claimed in one of the aforementioned claims is combined, together with suitable additives and excipients.

The present invention furthermore relates to a medicament prepared according to this process for the treatment of diseases associated with hdm2 protein, which contains at least one nucleic acid, at least one polypeptide or at least one antibody according to the present invention, if appropriate together with suitable additives and excipients. The invention

furthermore relates to the use of this medicament for the treatment of diseases associated with hdm2 protein.

The therapy of diseases associated with hdm2 protein can be carried out in a conventional manner, e.g. by means of infusions or injections which contain the medicaments according to the invention. The administration of the medicaments according to the invention can furthermore be optionally carried out in the form of liposome complexes or gold particle complexes. The treatment by means of the medicaments according to the invention can, however, also be carried out via oral dosage forms, for example tablets or capsules, via the mucous membranes, for example of the nose or the oral cavity, or in the form of dispositories implanted under the skin. TTS are known, for example, from EP 0 944 398 A1, EP 0 916 336 A1, EP 0 889 723 A1 or EP 0 852 493 A1. The (poly)peptides and their derivatives according to the invention can also be employed for the active and/or passive immunization of patients with diseases, in particular oncoses, which are connected with hdm2, in order to achieve the induction of, production of and increase in hdm-specific CTL and specifically to destroy the tumor and leukemia cells of the patients concerned. Such diseases include, for example, solid oncoses, lymphohematopoietic neoplasias, malignant hematological diseases, also in the form of a multiple myeloma (or plastocytoma), of a histiocytic lymphoma and of a CML myeloblastic crisis.

In a particularly preferred type of treatment, one or more cells, preferentially T-cells, in particular $CD8^+$-T-cells are taken from the patient, which are then transduced or transfected *ex vivo* with one or more genetic constructs according to the invention. The specific T-cells generated *ex vivo* can then subsequently be reimplanted into the patient. The process is thus similar to the immunotherapeutic

process described in Darcy et al. ("Redirected perforin-dependent lysis of colon carcinoma by ex vivo genetically engineered CTL" J. Immunol., 2000, 164:3705-3712) in the case of colon carcinomas using CTL transduced by scFv anti-CEA receptor, perforin and γ-IFN.

The present invention furthermore relates to a process for the production of a test for the discovery of functional interactors in connection with hdm2 protein-associated diseases, which is characterized in that at least one nucleic acid, at least one polypeptide or at least one antibody according to the invention is combined, together with suitable additives and excipients.

The term "functional interactors" within the meaning of the present invention is to be understood as meaning all those molecules, compounds and/or compositions and substance mixtures which can interact with the nucleic acids, polypeptides or antibodies according to the invention, if appropriate together with suitable additives and excipients, under suitable conditions. Possible interactors are simple chemical organic or inorganic molecules or compounds, but can also include peptides, proteins or complexes thereof. On account of their interaction, the functional interactors can influence the function(s) of the nucleic acids, polypeptides or antibodies *in vivo* or *in vitro* or alternatively only bind to the nucleic acids, polypeptides or antibodies according to the invention or enter into other interactions of a covalent or noncovalent manner with them.

The invention furthermore comprises a test produced according to the invention for the identification of functional interactors in connection with diseases associated with hdm2 protein, which contains at least one nucleic acid, at least one polypeptide or at least

one antibody according to the present invention, if appropriate together with suitable additives and excipients. Often, the pathological behavior of the cells *in vitro* can thus be imitated and substances can be sought which restore the normal behavior of the cells and which possess a therapeutic potential. Moreover, this test system can be utilized for the screening of substances which inhibit an interaction between the polypeptide according to the invention and a functional interactor.

A subject of the present invention is also a medicament for the indication and therapy of diseases associated with hdm2 protein, which contains a nucleic acid according to the invention or a polypeptide according to the invention and, if appropriate, suitable additives or excipients, and a process for the production of such a medicament for the treatment of diseases associated with hdm2 protein, in which a nucleic acid according to the invention or a polypeptide according to the invention is formulated with a pharmaceutically acceptable carrier.

Possible therapeutics and/or prophylactics are in particular vaccines, recombinant particles or injections or infusion solutions, which as active compound (a) contain the TCR-receptor polypeptide according to the invention and/or its derivatives and/or (b) a nucleic acid according to the invention and/or (c) T-lymphocytes produced *in vitro* or *ex vivo*, which contain a TCR specifically directed against hdm2.

For gene therapy application in humans, a medicament and/or recombinant particle is especially suitable which contains the nucleic acid according to the invention in naked form or in the form of one of the vectors active in gene therapy described above or in a form complexed with liposomes or gold particles. The pharmaceutical carrier is, for example, a physiological

buffer solution, preferably having a pH of about 6.0-8.0, preferably of about 6.8-7.8, in particular of about 7.4 and/or an osmolarity of about 200-400 milliosmol/liter, preferably of about 290-310 milliosmol/liter. Additionally, the pharmaceutical carrier can contain suitable stabilizers, such as, for example, nuclease inhibitors, preferably complexing agents such as EDTA and/or other excipients known to the person skilled in the art.

A further subject of the invention relates to a process for the preparation of a polypeptide for the diagnosis and/or treatment of diseases connected with hdm2 protein or for the identification of pharmacologically active substances in a suitable host cell, which is characterized in that a nucleic acid according to the invention is expressed in a suitable manner.

The polypeptide is thus prepared, for example, by expression of the nucleic acid according to the invention in a suitable expression system, as already described above, according to the methods generally known to the person skilled in the art. Suitable host cells are, for example, the *E. coli* strains DHS, HB101 or BL21, the yeast strain *Saccharomyces cerevisiae*, insect cell lines, e.g. from *Spodoptera frugiperda*, or the animal cells COS, Vero, 293, HaCaT, and HeLa, which are all generally obtainable.

A diagnostic according to the invention for therapy monitoring contains the polypeptide according to the invention or the immunologically active parts thereof described in greater detail above. The polypeptide or parts thereof, which are preferably bound to a solid-phase, e.g. of nitrocellulose or Nylon, can be brought into contact *in vitro* with the body fluid to be investigated, e.g. blood, in order thus to be able to react, for example, with autoimmune antibodies. The antibody-peptide complex can then be detected, for

example, with the aid of labeled anti-human-IgG or anti-human-IgM antibodies. The label is, for example, an enzyme, such as peroxidase, which catalyzes a color reaction. The presence and the amount of autoimmune antibodies present can thus be detected easily and rapidly by means of the color reaction.

Another diagnostic for therapy monitoring contains the antibodies according to the invention themselves. With the aid of these antibodies, for example, a tissue sample can be easily and rapidly investigated as to whether the polypeptide concerned is present in an increased amount, in order thereby to obtain an indication of diseases connected with hdm2 protein. In this case, the antibodies according to the invention are labelled, for example, with an enzyme, as already described above. The specific antibody-peptide complex can thereby be easily and likewise rapidly detected by means of an enzymatic color reaction.

A further diagnostic according to the invention comprises a probe, preferably a DNA probe, and/or primer. This opens up a further possibility of obtaining the nucleic acids according to the invention, for example by isolation from a suitable gene bank with the aid of a suitable probe (see, for example, J. Sambrook et al., 1989, Molecular Cloning. A Laboratory Manual 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, chapter 8 page 8.1 to 8.81, chapter 9 page 9.47 to 9.58 and chapter 10 page 10.1 to 10.67).

Suitable probes are, for example, DNA or RNA fragments having a length of about 100-1000 nucleotides, preferably having a length of about 200-500 nucleotides, in particular having a length of about 300-400 nucleotides, whose sequence can be derived from the polypeptides according to SEQ ID No. 1 to SEQ ID No. 8 of the sequence listing.

Alternatively, with the aid of the derived nucleic acid sequences oligonucleotides can be synthesized which are suitable as primers for a polymerase chain reaction. Suitable primers are, for example, DNA fragments having a length of about 10-100 nucleotides, preferably having a length of about 15 to 50 nucleotides, in particular having a length of 20-30 nucleotides, whose sequence can be derived from the polypeptides according to SEQ ID No. 1 to SEQ ID No. 8 of the sequence listing.

The term "coding nucleic acid" relates to a DNA sequence which codes for an isolable bioactive polypeptide according to the invention or a precursor. The polypeptide can be encoded by a sequence of full-length or any part of the coding sequence, as long as the specific, for example enzymatic, activity is retained.

It is known that small changes in the sequence of the nucleic acid according to the invention can be present, for example due to the degeneration of the genetic code, or that nontranslated sequences can be appended at the 5' and/or 3'-end of the nucleic acid without its activity being significantly changed. This invention therefore also comprises "functional variants" of the nucleic acids according to the invention.

By the term "functional variants", all DNA sequences are denoted which are complementary to a DNA sequence, which hybridize under stringent conditions with a derived reference sequence or parts thereof, in particular the hypervariable V(D)JC region, and have a similar or identical activity to the corresponding polypeptide according to the invention.

"Stringent hybridization conditions" are to be understood as meaning those conditions under which a hybridization takes place at 60°C in 2.5 x SSC buffer,

followed by a number of washing steps at 37°C in a lower buffer concentration, and remains stable.

The term "functional variants" within the meaning of the present invention is understood as meaning polypeptides which are related functionally to the polypeptides according to the invention, i.e. have structural features of the polypeptides. Examples of functional variants are the corresponding polypeptides which originate from organisms other than the mouse, that is the human, or, preferably from nonhuman mammals such as, for example, monkeys, pigs and rats. Other examples of functional variants are polypeptides which are encoded by different alleles of the gene, in various individuals or in various organs of an organism. The present invention in particular also covers functional TCR variants, which recognize the identical epitope of the hdm2 polypeptide and induce a specific T-cell response.

In a further sense, these are also understood as meaning polypeptides which have a sequence homology, in particular a sequence identity, of about 70%, preferably about 80%, in particular about 90%, especially about 95% to the polypeptide having the amino acid sequence according to one of SEQ ID No. 1 to SEQ ID No. 8 and/or to DNA sequences derived with the aid of the peptide sequences. Among these are also included additions, inversions, substitutions, deletions, insertions or chemical/physical modifications and/or replacements or parts of the polypeptide in the range from about 1-60, preferably from about 1-30, in particular from about 1-15, especially from about 1-5 amino acids. For example, the first amino acid can lack methionine without the function of the polypeptide being significantly modified.

The invention will now be illustrated further with the aid of the attached examples and figures, without being restricted by this. The figures show:

SEQ ID No. 1: polypeptide of the wild-type gene mouse αTCR (muvα-mucα),

SEQ ID No. 2: polypeptide of the wild-type gene mouse βTCR (muvβ-mucβ),

SEQ ID No. 3: polypeptide of a chimeric partially humanized TCR gene (muvα-hucα),

SEQ ID No. 4: polypeptide of a chimeric partially humanized TCR gene (muvβ-hucβ)

SEQ ID No. 5: polypeptide of a single-chain TCR muvα-Li-muvβ-mucβ,

SEQ ID No. 6: polypeptide of a single-chain TCR muvβ-Li-muvα-mucα,

SEQ ID No. 7: polypeptide of a double-chain TCR in fusion with human CD3ζ chain muvα-hucα-huCD3ζ,

SEQ ID No. 8: polypeptide of a double-chain TCR in fusion with human CD3ζ chain muvβ-hucβ-huCD3ζ,

SEQ ID No. 9: primer for_bTCR_v4,

SEQ. ID No. 10: primer rev_bTCR_c4,

SEQ. ID No. 11: primer for_Eco_bTCR_v5,

SEQ. ID No. 12: primer rev_Asc_bTCR_c6,

SEQ. ID No. 13: primer for_Eco_bTCR_c2,

SEQ. ID No. 14: primer rev_Asc_bTCR_c2,

SEQ. ID No. 15: primer rev_Asc_aTCR_c1,

SEQ. ID No. 16: primer rev_aTCR_c5,

SEQ. ID No. 17: primer rev_Asc_aTCR_c4,

SEQ. ID No. 18: primer for_Eco_aTCR_c6,

SEQ. ID No. 19: primer for_NcoI_aTCR_4,

SEQ. ID No. 20: primer for_NcoI_aTCR_5b; phosphorylated at the 5'-end,

SEQ. ID No. 21: primer for_NcoI_bTCR_4,

SEQ. ID No. 22: primer for_NcoI_bTCR_5b; phosphorylated at the 5'-end,

SEQ. ID No. 23: primer rev_aTCR_BamHI_1,

SEQ. ID No. 24: primer rev_bTCR_BamHI_1,
SEQ. ID No. 25: primer for_hucaTCR_2,
SEQ. ID No. 26: primer rev_hucaTCR_2,
SEQ. ID No. 27: primer for_hucaTCR_AlwNI_3,
SEQ. ID No. 28: primer rev_hucaTCR_BamHI_1,
SEQ. ID No. 29: primer for_hucbTCR_3,
SEQ. ID No. 30: primer rev_hucbTCR_2,
SEQ. ID No. 31: primer for_hucbTCR_4; phosphorylated at the 5'-end,
SEQ. ID No. 32: primer rev_hucbTCR_BamHI_1,
SEQ. ID No. 33: primer T7_for,
SEQ. ID No. 34: primer T7_rev,
SEQ. ID No. 35: primer rev_hZeta_BamHI_4,
SEQ. ID No. 36: primer rev_huca-hu_tm_Zeta,
SEQ. ID No. 37: primer rev_hucb-hu_tm_Zeta,
SEQ. ID No. 38: polynucleotide of the wild-type gene mouse αTCR (muvα-mucα) from SEQ ID No. 1,
SEQ. ID No. 39: polynucleotide of the wild-type gene mouse βTCR (muvβ-mucβ) from SEQ ID No. 2,
SEQ. ID No. 40: polynucleotide of the chimeric partially humanized TCR gene (muvα-hucα) from SEQ ID No. 3,
SEQ. ID No. 41: polynucleotide of the chimeric partially humanized TCR gene (muvβ-hucβ) from SEQ ID No. 4,
SEQ. ID No. 42: polynucleotide of the single-chain TCR muvα-Li-muvβ-mucβ from SEQ ID No. 5,
SEQ. ID No. 43: polynucleotide of the single-chain TCR muvβ-Li-muvα-mucα from SEQ ID No. 6,
SEQ. ID No. 44: polynucleotide of the double-chain TCR in fusion with human CD3ζ chain muvα-hucα-huCD3ζ from SEQ ID No. 7; and
SEQ. ID No. 45: polynucleotide of the double-chain TCR in fusion with human CD3ζ chain muvβ-hucβ-huCD3ζ from SEQ ID No. 8.

In the primer sequences, Y=C/T;R=A/G;V=A/C/G;W=A/C/T

Figure 1 shows a FACS analysis (see 1.4) by means of a vβ6-specific FITC-labeled antibody (Pharmingen). Both the murine T-cell line and the clone 3 resulting therefrom were checked. The isotype control used was anti-TCR Vα2 - FITC (BD), the positive control the anti-pan βTCR - FITC -antibody (BD). The negative control used was the influenza matrix protein-specific T-cell clone 58.

Figure 2 shows a vβ6-specific antibody blockade of the murine T-cell clone 3 in the cytotoxic ^{51}chromium release test (see 1.4). The blockade was evaluated dose-dependently. The negative control used was, on the one hand, an anti-Vα3.2 antibody (BD) and, on the other hand, the p53 - 264-272 - specific T-cell clone 46, which was crosschecked against its individual recognizing p53 peptide. The TAP-deficient cell line T2, which was loaded exogenously with 10^{-8} M peptide, functioned as a target cell.

Figure 3 shows examples of the design of hdm2-specific constructs at the DNA level (see 2.), where muV α/β is murine variable α/β gene segment; muC α/β is murine constant α/β gene segment; huC α/β is human constant α/β gene segment; TM: gene segment coding for trans-membrane; Li: gene segment coding for $(GGGGS)_3$ motif and HuCD3ζ is human CD3ζ-chain gene segment. The restriction cleavage sites relevant for the cloning are indicated.

Figure 4 shows the same examples of the design of hdm2-specific constructs at the protein level (see 2.), where muV α/β is murine variable α/β domain; muC α/β is murine constant α/β domain; huC α/β is human constant α/β domain; SP: murine wild-type signal peptide; TM: transmembrane; Li is linker with $(GGGGS)_3$ motif and huζ: humane CD3ζ-chain with 3 consecutive ITAM motifs.

The numbers derived from the bacterial clones are indicated for the individual chains.

Figure 5 shows the polylinker of the construct muvα-Li-muvβ-mucβ (see 2.3). The polylinker has finally been inserted into the resulting construct by means of *Kpn* I und *Dra* III.

Figure 6 shows the polylinker of the construct muvβ-Li-muvα-mucα (see 2.3). The polylinker has finally been inserted into the construct by means of *Sca* I und *Bsg* I.

Figure 7 shows the fusion site of the chimeric double-chain TCR in fusion to the human CD3ζ-chain muvα-hucα-huCD3ζ (see 2.4). The extracytosolic cysteine dimerizing via a disulfide bridge is indicated by relative position to the start methionine.

Figure 8 shows the fusion site of the chimeric double-chain TCR in fusion to the human CD3ζ-chain muvβ-hucβ-huCD3ζ (see 2.4). The extracytosolic cysteine dimerizing via a disulfide bridge is indicated by relative position to the start methionine.

Figure 9 shows the "fluorescence activated cell sorting" (FACS) analysis of the constructs described under 2.) after retroviral transduction. Examples are shown of average transduction efficiencies for OKT3-activated PBL cultures 6 days after transduction. These are in the range from 10-25% of the $CD3^+$ lymphocytes employed. The negative control used was a transfection/transduction batch containing the empty retroviral vector pBullet.

Figures 10 to 12 show peptide titrations (see 3.4.1) where T2 target cells were loaded exogenously with the hdm2-81-88 peptide in a concentration series from 1 x 10^{-6}M to 1 x 10^{-12}M. The reference substance used was the

murine T-cell clone 3 hdm2-81-88 (synonym: P2) of the huCD8 x $A2K^b$ transgenic mouse. The ^{51}chromium release tests shown were carried out using transduced PBLs, which were enriched to over 90% of $v\beta6^+$ T-cells by means of vβ6-directed magnetic cell-sorting (Miltenyi-Biotec). Figure 10: the negative control used was a construct of a nonfunctional single-chain T-cell receptor (nfTCR), which although it could be expressed comparatively well on the surface of the T-cells and thereby could also be enriched by means of magnetic cell sorting, showed no functionality in the CTL test. Figure 11: peptide titration of the murine heterodimeric wild-type construct muTCR/8-18. Figure 12: peptide titration of the chimeric heterodimeric construct huTCR/10-29.

Figures 13 to 15 show the recognition of malignant transformed cell lines. The target cells chosen were HLA-A2$^+$. The reference used was the murine T-cell clone 3 hdm2-81-88 (synonym: P2) of the huCD8 x $A2K^b$ - transgenic mouse. The ^{51}chromium release tests shown were carried out using transduced PBLs which were enriched to over 90% of $v\beta6^+$-T-cells by means of vβ6-directed magnetic cell sorting (Miltenyi-Biotec). Figure 13: the negative control used was a construct of a nonfunctional single-chain T-cell receptor (nfTCR), which although it could be expressed comparatively well on the surface of the T-cells and thereby could also be enriched by means of magnetic cell sorting, showed no functionality in the CTL test. Figure 14: murine heterodimeric wild-type construct muTCR/8-18. Figure 15: chimeric heterodimeric construct huTCR/10-29.

Examples

The genes of the T-cell receptors αTCR and βTCR from a double-transgenic mouse huCD8 x $A2K^b$ were prepared which mediate an HLA-A2.1-restricted and hdm2 protein / 81-88 peptide-specific T-cell response. The genes obtained

were inserted retrovirally into human peripheral blood lymphocytes (PBLs) as wild-type (WT) or modified constructs and the HLA-restricted antigen recognition was checked functionally by means of CD8-mediated cytotoxic lysis of various cell lines in the ^{51}chromium release test.

1.) Obtainment of the genes coding for the α- and β-chains of the T-cell receptors

1.1) Praparation of the cytoplasmic RNA

The cytoplasmic RNA was isolated, on the one hand, specifically to separate off the still unprocessed, intron-containing RNA, DNA fragments and ribosomal RNA, which are located mainly in the cell nucleus, and to achieve an enrichment of the desired "messenger" RNA. The protocol and materials correspond largely, with small modifications, to the "Rneasy Mini Kit" from QIAGEN: for one preparation, on average 50×10^6 murine cells of the hdm2/81-88 -specific T-cell clone 3 from the culture dishes of a "24-well plate" were combined and washed serum-free in RPMI 1640 (Biowhittaker). The cells were aliquoted under RNase-free conditions at 12.5×10^6 cells each per incubation tube and incubated in a cell membrane lysis buffer (4°C-cooled) for 20 min on ice:

Cell membrane lysis buffer:

50 mM tris HCl pH 8.0
140 mM NaCl
1.5 mM $MgCl_2$
0.5 % Nonidet P40 (Roche 1 332 473)
1000 units of Rnase inhibitor/ml ZML-P (Roche 799 017)
1 mM DTT

For this buffer, like all other aqueous solutions which have to be prepared, DEPC-treated and double-autoclaved deionized (MilliQ from Millipore) water was used.

The further steps were carried out according to a process familiar to the person skilled in the art with the aid of the QIAGEN protocol under Rnase-free conditions at room temperature and are described briefly:

The gently solubilized and aliquoted cells were centrifuged at 300g / 2' (minutes) / 4°C and the supernatant was removed. 600 µl of a β-mercaptoethanol (MSH)-containing (1.45 mM) "RLT" buffer were added, well mixed and 430 µl of 96% ETOH were added with mixing. The solution per aliquot was added to a silica Minispin column and centrifuged for 15" (seconds) at 10,000 rpm (revolutions per minute) in an Eppendorf table centrifuge. A wash in 700 µl of "RW1" and in 500 µl of 77% ETOH-containing "RPE" buffer was in each case carried out. Finally, 500 µl of identical "RPE" buffer were added and the column was dried for 2' at 14,000 rpm maximum. The RNA was eluted using 54 µl of Rnase-free water at 10,000 rpm for 2'. The yields were 30µg of RNA per 12.5×10^6 murine T-cells. The RNA was frozen down at 6 µg /10 µl at -20°C.

1.2) Amplification of the β-chain gene by means of coupled RT-PCR

The TCR-coding RNA had to be specifically transcribed into DNA and amplified before it was possible to clone it. For this, RT-PCR was methodically used, which is implemented in the like-named kit "Titan One Tube RT-PCR System" from Roche (1888 382). The "one-step protocol" was chosen, in which the reverse transcription is followed without further intervention of the polymerase chain reaction. The enzymes used were the AMV reverse transcriptase and a mixture of the *Thermus Aquaticus / Pwo* - thermostable polymerase ("Expand™ High fidelity" enzyme mix), which was used in a universal reaction buffer. Since the transcription rate of the TCR genes and, on account of the variance of the TCR genes, the conservation of the gene

sequences coding for the variable domains is low, a "nested PCR" (polymerase chain reaction) was added in order to increase the specificity and the signal strength of the amplified DNA. The variability of the v-gene segment, whose subfamily membership it was not possible to predict a priori, was attempted to determine using a degenerate primer (**for_bTCR_v4; Seq ID No. 9**), which corresponded to a base sequence for the first conserved amino acids coding after the signal peptide. An artificial gene segment, which codes for a murine TCR signal peptide, should be added primarily by means of synthetic oligonucleotides in order to guarantee a direction of the protein synthesized *in vivo* into the lumen of the endoplasmic reticulum. A reverse-hybridizing primer (**rev_bTCR_c4; Seq ID No. 10**) within the constant gene segment was chosen in order to avoid the additional variance of the two possible c-gene segments (c-β1 or c-β2) of the β-chain, which have some base differences at their 3'-end. The oligonucleotides of the "nested PCR" possessed, besides the TCR complementarity, additionally at its 5'-end sequences of the restriction cleavages sites for *Eco*RI or *Asc*I. The primer (**for_Eco_bTCR_v5; SeqID No. 11**) hybridizing in the forwards direction of the nested "PCR" was isometric in the sequence of the v-gene segment to the degenerate RT-PCR primer, whereas the primer (**rev_Asc_bTCR_c6; SeqID No. 12**) complementary to the c gene segment hybridizing in the backwards direction was moved inwards more than 120 bases relative to the reverse 1st primer of the TCR reading frame, such that strictly a "3'-located nested PCR" was involved. By means of this, the specificity of the PCR was increased, since in 2 independent PCR steps at 2 different locations a sequence-specific recognition event must have taken place. The complete c-gene segment was included and cloned by means of a reverse degenerate primer (**rev_Asc_bTCR_c2; SeqID No. 14**) for the inclusion of the two potentially possible cβ1/cβ2 gene segments and of a corresponding primer

(**for_Eco_bTCR_c2; SeqID No. 13**) in the reading direction of the cβ gene segment by means of RT-PCR without added "nested PCR". In the overlapping range of the two amplificates in the cβ range, a unique *Nco*I cleavage site was situated, by means of which it was possible to combine the truncated vβ/cβ and the complete cβ gene segment in a subsequent subcloning.

The pipetting scheme and the PCR protocol of the RT-PCR corresponded in principle to the process described by Roche. The subsequent "nested PCR" was carried out analogously with replacement of the components Rnase inhibitor and DTT by autoclaved water. Instead of the 2 µl of RNA (0.1 -1 µg), 2 µl of the amplificate of the RT-PCR was employed. The technical requirements of the PCR apparatus made possible the simultaneous test of various hybridization temperatures in the 2nd step of the classical PCR cycle in a temperature gradient from 35°C - 55°C in intervals for 5 selected temperatures of 36°C, 39°C, 44°C, 51°C and 56°C. For the higher temperatures from 44-56°C, it was possible to achieve single bands of the desired length, whose intensity were increased by the 2nd PCR and were sufficient in order to be inserted into vectors.

The sequencing of 8 bacterial clones showed some randomized base replacements, which were based on the comparatively high error rate of the Expand™ enzyme mix to other polymerases. The clones chosen moreover all carried the sequence information for the signal peptide and a short region of the noncodogenic, 5'-untranslated region of less than 100 bases, such that it was possible to assume from this that the v-gene segment-specific degenerate primer recognized a similar sequence upstream and yielded the additional sequence of the wild-type signal peptide.

1.3) Amplification of the α-chain gene by means of RACE-PCR

For the sequence determination of the α-chain, RACE-PCR was the strategy leading to the target:
the degenerate oligonucleotide designed analogously to the batch described above exclusively afforded the codogenic sequence of a T-cell receptor which is nonfunctional on account of a reading frame shift in the CDR3 loop. The allele exclusion is not valid to the same extent as for the β-chain of the TCR. The RACE technology is established in the like-named system of Roche (1 734 792) and must be supplemented with αTCR gene-specific oligonucleotides: for this, in the context of the "5'-RACE-PCR", the 5'-located variance of the codogenic sequence is circumvented by generating a known, short adenine oligonucleotide sequence at the 3'-end of the reverse-transcribed cDNA sequence by means of the terminal transferase, which as a result functions as a recognition sequence for a PCR primer 5'-located in the reading direction. The reverse PCR primers lie within the invariant sequence, which codes for the constant domain. For the reverse transcription, the primer **rev_Asc_aTCR_c1 (SeqID No. 15)** was used, which together with **for_Eco_aTCR_c6 (SeqID No. 18)** was also used for the amplification via RT-PCR of the full-length cα segment. The concept of a consecutive 2-step "nested PCR" with the aid of the primer pair "Oligo d(T)-anchor primer" (component of the RACE-PCR kit) / **rev_aTCR_c5 (SeqID No. 16)** and "PCR anchor primer" (component of the RACE-PCR kit) / **rev_Asc_aTCR_c4 (SeqID No. 17)** was followed, so that here too the subcloning of the complete sequence, which codes for the constant domain, was added. The PCR primers had to lie such that the single *Nco*I cleavage site lay in the overlapping region of the amplified gene sections. The truncated PCR amplificate containing the vα gene segment was cloned into pNEB193 (New England Biolabs) by means of 5'-*Sal*I and 3'-*Asc*I, whereas the constant gene segment was inserted into pNEB193 by means of *Eco*RI and *Asc*I. The amplificates were cloned conventionally (J. Sambrook et al., 1989, Molecular

Cloning. A Laboratory Manual 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor) by means of purification through LMP gels and phenol/chloroform-extraction, before they were digested by restriction enzymes and the ends were removed with the aid of the "High Pure PCR Product Purification Kit" (Roche, 1 732 668).

The multistep protocol largely follows the commercial protocol with the aid of a programmable PCR apparatus: the reverse transcription, the transferase reaction and the subsequent 2-step "nested PCR" were carried out in the PCR apparatus "MasterCycler" from Eppendorf. In the context of the specific α-chain amplification, gradient technology was used in the hybridization step for the temperatures 39°C, 44°C, 52°C and 56°C. The *Pfu* polymerase (Stratagene) was chosen, since at this point in time this was regarded as the enzyme having the lowest error incorporation rate. The 1st PCR already afforded a weak band of the size to be expected of 0.9 kB in the overall temperature spectrum, whose intensities were increased by the subsequent PCR and moreover it was possible to dilute out "primer dimers" which occurred. In the final PCR, a truncation of the amplificate by 70 bp resulted according to the theoretical position of the moved hybridizing primer. In the agarose gel, a double band of 100 bp length difference stood out, which pointed to the presence of the functional and the nonfunctional α-chain. The bands were jointly excized in the preparative gel in order to be able to include both amplificates in the subsequent cloning. 7 clones were found, which coded for a functional reading frame and possessed identical sequences.

1.4) Checking of the subfamily memberships found

The existence of a vβ6- or vα10-gene segment in the murine hdm2 protein/ 81-88-specific T-cell clone 3 was verified in various ways:

a) RT_PCR by means of a vβ6-specific primer or of a vα10-specific primer. The RNA was purified beforehand from the murine hdm2-P2-specific T-cell clone 3 by separating the specific T-cells from the murine "feeder" cells by means of anti-huCD8 magnetically labeled antibodies. The negative control used was an influenza matrix protein-(FluM1 58-66)-specific T-cell clone.

b) FACS analysis (**Figure 1**) by means of a vβ6-specific FITC-labeled antibody (Pharmingen). Both the murine T-cell line and the clone 3 resulting therefrom were checked. The positive control used was an FITC-labeled antibody which recognizes the β-chain portion independently of the subfamily.

c) vβ6-specific antibody blockade of the murine T-cell clone 3 in the cytotoxic ^{51}chromium release test (**Figure 2**).

2.) Examples of the design of hdm2-specific constructs (Figure 3 [SEQ ID Nos 38-45] und 4 [SEQ ID Nos 1-8])

The retroviral vector pBullet, a functional derivative of pStitch (Weijtens et al., 1998) belongs to the "splice vectors", in which the transgene must be cloned in the same sequence context relative to the splice acceptor and donor elements SA and SD as the *env* gene in order to guarantee maximum expression. For this, an *Nco*I cleavage site is designed within the start codon "ccATGg" by means of appropriate primers. This changed, for both wild-type chains αTCR and βTCR, the 2nd amino acid from Lys to Glu or Asn to Asp. At the 3'-end of the reading frame, the unique *Bam*HI-cleavage site was chosen. The restriction enzymes and modifying enzymes were obtained from New England Biolabs (NEB). The thermostable polymerase was generally the native or cloned *Pfu* DNA polymerase (*Pyrococcus furiosus*, Stratagene) or the Pfx DNA polymerase (*Pyrococcus sp.*, Life Technologies)

2.1) Cloning of the murine wild-type genes αTCR (muvα-mucα; SeqID Nos. 1-38) and βTCR (muvβ-mucβ; Seq ID Nos. 2-39)

For the case where the *Nco*I cleavage site did not exist internally (partially humanized chains), it was possible to amplify and clone the gene segments directly by means of primers which contained the sequences for *Nco*I **(for_NcoI_aTCR_4, SeqID No. 19; for_NcoI_bTCR_4, SeqID No.21)**.

Since the *Nco*I-cleavage site for the wild-type chains occurred in the gene segment for the constant domains, the codogenic reading frames were amplified by means of oligonucleotides **(for_NcoI_aTCR_5b, SeqID No. 20; for_NcoI_bTCR_5b, SeqID No. 22)**, which, 5'-terminally, possessed truncated, blunt-ended *Nco*I cleavage sites of this type, so that these, after blunt-ended filling of *Nco*I-cleaved vector DNA with the aid of the T4 DNA polymerase, complement with the latter to give complete *Nco*I cleavage sites. **rev_aTCR_BamHI (SeqID No. 23)** and **rev_bTCR_BamHI_1 (SeqID No. 24)** were used as final reverse primer for all murine double-chain and single-chain constructs.

2.2) Generation of the chimeric, partially humanized TCR genes huαTCR (muvα-hucα; SeqID No. 40) and huβTCR (muvβ-hucβ; SeqID No. 41)

For a functional chimerization of murine TCRs, 2 criteria should be fulfilled:

a) the murine constant domains should be replaced by the corresponding human domains in such a way that these domains are completely included in their immunoglobulin-like folding structure. The complete variable domains were likewise to be included in order to cause no impairment of the almost carboxy-terminal lying CDR3 loop.

b) The fusion had to take place in such a way that the transition if possible generated no neoantigen

which could lead to an unintentional immune reaction.

In the case of b), in general for both chains in comparison of human with murine sequences the following properties resulted: in a region of up to 8 amino acids behind the last consensus motif "GxG", which completes the CDR3 loop, the positions are relatively strongly conserved. This region is completed by a highly conserved sequence of up to 11 amino acids. After this, the murine sequences begin to differ from the human sequences, for each species highly conserved. The central region lies in the extended loop which connects the variable domain with the constant domain (see criterion a)). In the ideal case, the chimerization within a highly conserved amino acid (AA) triplet sequence was to be preferred.

In the case of the αTCR, the highly conserved triplet sequence consisted of "Q-N-P" (base 400-408 relative to the start codon of the murine αTCR). In position 407-415 was attached a unique *Alw*NI cleavage site, which it was possible to use for fusion: the 3'-shifted position or the *Alw*NI demanded that, instead of the aspartate conserved in human sequences, the glutamate conserved in murine sequences was present. This conservative amino acid replacement was considered as not very immunogenic and thus tolerable. The chimeric, highly conserved region read I^{133}-Q-N-P-**E**-P-A-V-Y-Q-L-**R**^{144} in which R^{144} represents the first human-specific amino acid. I^{133} is situated in the ninth position behind the consensus motif "GxG" completing the CDR3 loop.

The cloning strategy planned ligating the LMP-agarose-isolated *Eco*RI/*Alw*NI-cleaved vα segments with the *Alw*NI/*Bam*HI-*digested* cα PCR amplificates, subsequently cleaving the mixed ligation products using *Eco*RI and *Bam*HI and cloning the prepared ligation product of the potentially correct fragment length into the expression

vector pcDNA3.1(-) (Invitrogen). An asymmetric test digestion afforded the clones having the desired sequence of the ligation starting materials.

The human constant gene segments cα and cβ were isolated from the human leukemia cell line Jurkat by conventional RT-PCR, as is implemented as part of the Roche kit described above, with subsequent "nested" PCR.

In the case of the human cα gene segment, with the aid of mutagenic PCR primers in 2 positions, on the one hand the *Alw*NI cleavage site **(T** to **A; see table below)** had to be generated and on the other hand the compatibility with the murine sequence had to be produced (**c** to **a; see table below**), since the middle 3 positions of the *Alw*NI cleavage site may be degenerate:

AA	Q[134]			N			P			E/D			P			A[139]		
Murine	C	A	G	A	A	C	C	C	A	G	a	a	c	C	T	G	C	T
Human	C	A	G	A	A	C	C	C	T	G	a	c	c	C	T	G	C	C

The *Alw*NI cleavage site occurring naturally in the murine sequence is shown in bold, the 3'-shifted cleavage position is underlined. The degenerate triplet is typed in lower case. The base replacements necessary for the human "primer" for the retention of a mouse sequence-compatible *Alw*NI cleavage site are shown in bold. The 5' initial base of the mutagenic primer is in italics. The resulting chimeric AA sequence is shown in bold (see text); the highly conserved AA triplet is moreover underlined.

The amplification of the cα fragment was carried out by means of a "nested" PCR. The mutations were placed in the "nested" primer (**for_hucaTCR_AlwNI_3; SeqID No. 27**), whereas the RT-PCR primer (**for_hucaTCR_2; SeqID No. 25**) still corresponded to the human wild-type

sequence. The reverse "nested" primer **rev_hucaTCR_BamHI_1** (**SeqID No. 28**) possessed both the stop codon and the clonable *Bam*HI cleavage site, whereas the reverse RT-PCR primer (**rev_hucaTCR_2; SeqID No. 26**) recognized the 3'-lying noncodogenic region.

In the case of the βTCR, the "nested" PCR principle coupled with a blunt-ended ligation was likewise used (**SeqID Nos. 29-32**). The highly conserved triplet sequence consisted of "E-D-L" (base 400-408 relative to the start codon of the murine βTCR). A *Bst*YI cleavage site comprising the variable gene segment and which was unique was located isometrically (base 402-407). A primer specific for the human constant domain gene segment (**for_hucbTCR_4; SeqID No. 31**) should complement via its 5'-terminal end, which with a phosphorylated "T" represents the last base of the potentially palindromic recognition sequence of *Bst*YI, the with *Bst*YI-digested (produces overhanging 5'-ends which are filled with the T4-DNA polymerase complementary with the addition of dNTP's and are not degraded) and blunt-ended filled 3'-end of the murine variable domain gene segment, such that the correct reading frame context is retained. The vβ fragment was subsequently cleaved using *Eco*RI and isolated from an LMP gel, the cβ PCR amplificate was employed in the ligation as an additionally enzymatically phosphorylated (T4-poly-nucleotide kinase, NEB) unilaterally blunt-ended *Bam*HI fragment. The mixed ligation products were subsequently cleaved using *Eco*RI / *Bam*HI and the fragment having the potentially correct fragment length cloned into pcDNA3.1(-). An asymmetric test digestion showed the clones having the desired sequence and orientation of the ligation starting materials.

AA	**E^{133}**			**D**			**L**		
murine	G	A	**G**	**G**	**A**	**T**	**C**	**T**	*G*
human	G	A	G	G	A	*C*	C	*T*	*G*

The *BstYI* cleavage site occurring naturally in the murine sequence is shown in bold, the 5'-shifted cleavage position, which generates 5'-overhangs, is underlined. The 5' initial and phosphorylated base "T" of the human "nested" primer, which complement the last base of *BstYI* in the chimeric construct, is in italics. The resulting chimeric AA sequence is shown in bold (see text); the highly conserved AA triplet is moreover underlined.

The chimeric highly conserved region read E^{133}-D-L-**N**^{136} in which N^{136} represents the first human-specific amino acid. E^{133} is located in the seventh position behind the conserved "GxG" motif, which completes the CDR3 loop.

2.3) Generation of the single-chain TCRs muvα-Li-muvβ-muccβ (SeqID No. 42) and muvβ-Li-muvα-muccα (SeqID No. 43)

Single-chain TCRs consist of the fusion of the variable domains vα with vβ and of an added constant domain cα or cβ (**Eshar et al., 1993**). The advantage of such constructs lies in the defined 1:1 stoichiometry of the two variable domains in a construct and the defined reactivity on account of the heterogenous pairing of endogenous with exogenous double-chain TCRs which is to be excluded. The variable domains were connected via a flexible polylinker of a redundant $(GGGGS)_3$ motif. The ends of the synthetic oligonucleotide accommodated unique cleavage sites, with which it was possible to connect the carboxy-terminal end of the one variable domain, placed behind the essential CDR3 loop, with the beginning of the other variable domain, lying behind the sequence which codes for the signal peptide. Conceptionally, five criteria were taken into consideration:

a) the polylinker had to be long enough in order to connect the ends of the variable ends. For this, murine TCR crystal structures of a MHC-H-$2K^b$ specificity were consulted.

b) the polylinker had to connect the ends in such a way that these, on the one hand, did not adversely affect the specificity of the CDR3 loop, on the other hand adequately eliminated the signal peptide of the added variable domain with retention of its functional structure. In order to bring this about, amino acid sequences of the respective variable domain were additionally contained in the polylinker for the case in which the unique cleavage sites did not lie ideally.

c) The polylinker had, on the one hand, to be flexible, which should be brought about with the aid of a sterically favorable catenation of glycine residues, on the other hand be sufficiently soluble in order to avoid aggregate formation and precipitation: the hydroxyl group of the serine residue forms hydrogen bonds with solvating water molecules and forms local dipoles.

d) in order not to adversely affect the transcription in the correct reading frame, the codogenic triplets for the glycine residues were varied ("GGC" and "GGA").

e) the polylinker had to possess the same internal unique cleavage sites as the wild-type sequences in order to cleave the intermediates in a 2-step cloning process and to add the almost complete wild-type sequences 3'-terminally via these unique cleavage sites.

Under these conditions, it was possible to allow two orientations of the variable domain:

In the case of the construct **muvα-Li-muvβ-mucβ** (**SeqID No. 42; Figure 5**), the unique cleavage site *Kpn*I behind the last consensus motif "GxG" of the variable vα domain and *Dra*III was chosen as the restriction cleavage site concluding the signal peptide coding sequence in the vβ domain. In a first step, the hybridized, double-stranded oligonucleotide was cleaved bilaterally using *Kpn*I and cloned between the vα-coding

and cα-coding sequence of the wild-type αTCR. After this, the chimeric intermediate was cleaved using *Dra*III/*Bam*HI and the the βTCR-coding sequence, which had lost its signal peptide-coding sequence by means of a *Dra*III/*Bam*HI digestion, was cloned in. The first glycine of the polylinker lay in position 6 relative to the amino-terminal Gly of the "GxG" consensus motif in vα, the last serine of the polylinker, on account of an interposed blunt-ended ligation connected via an arginine, before position Asp^{22} of the vβ domain.

In the case of the construct **muvβ-Li-muvα-mucα (SeqID No. 43; Figure 6)**, the unique cleavage site *Sca*I behind the last consensus motif "GxG" of the variable vβ domain and BsgI was chosen as the restriction cleavage site concluding the signal peptide coding sequence in the vα domain. In a first step, the hybridized, double-stranded oligonucleotide was cleaved bilaterally using *Sca*I and cloned between the vβ-coding and cβ-coding sequence of the wild-type βTCR. After this, the chimeric intermediate was cleaved using *Bsg*I/*Bam*HI and the the αTCR-coding sequence, which had lost its signal peptide-coding sequence by means of a *Bsg*I/*Bam*HI digestion, was cloned in. The first glycine of the polylinker lay in position 6 relative to the amino-terminal Gly of the "GxG" consensus motif in vβ, the last serine of the polylinker before position Val^{24} of the vα domain.

2.4) Generation of the double chain TCRs in fusion to the human CD3ζ-chain muvα-hucα-huCD3ζ (SeqID No. 44; Figure 7) and muvβ-hucβ-huCD3ζ (SeqID No. 45; Figure 8)

The signal transduction of the double-chain TCRs should be optimized. For this, the respective double-chain α/βTCRs were fused to the almost complete CD3ζ subunit of the CD3 complex. This fusion brings about, according to various publications **(Eshar et al., 1994)**, an efficient and CD3-independent coupling of the TCR to

the effectors Lck[56] or ZAP-70 lying upstream or downstream in the cascade.

A modified ligation PCR-based technique was established, with the aid of which independently of unique cleavage sites it was possible to produce any desired fusions for the preparation of chimeric proteins and the specificity of the PCR amplificates obtained was markedly increased in comparison to a conventional ligation PCR. The genetic fusion is defined by means of a reverse, chimeric primer **(rev_huca-hu_tm_Zeta, SeqID No. 36, Figure 7; rev_hucb-hu_tm_Zeta; SeqID No. 37, Figure 8)**, which with a forwards primer **(T7_for; SeqID No. 33)** as a PCR amplificate produces the "mega-primer": in a first, conventional ligation PCR step, for the 5'-lying fusion partner a "megaprimer" is generated, which moreover carries a short sequence specific for the 3'-lying fusion partner as a sequence appendage. In order markedly to increase the specificity of the PCR signals, as early as in the first PCR primers are used which hybridize outside the codogenic, chimeric amplificate, on the initially introduced "templates" **(T7_for; T7_rev; SeqID No. 33,34)**, in order then to use inwards-lying ("nested") primers in the second PCR. In this, the mega-primer yields, together with a reverse "nested" primer **(rev_hZeta_BamHI_4; SeqID No. 35)** hybridizing to the 3'-lying fusion partner, an intermediate product which, with a third forward primer **(for_aTCR_NcoI_5b; for_bTCR_NcoI_5b; SeqID Nos 20, 22)**, lying "nested" at the 5'-end, yields a PCR amplificate trimmed at the ends.

The fusion point was restricted to the cysteines forming the disulfide bridges: by this means the TCRs lost their cytoplasmic sequence, the transmembranes and a short region of amino acids lying extra-cytoplasmically up to the dimerizing cysteine, the human □-chain likewise lost a short extracytoplasmic

section, but exclusive of its cystine-forming cysteine should code for the transmembrane and cytosolic region including the three bivalent "ITAM" motifs. The reverse chimeric primer codes for the 21 bases lying 3'-terminally of the respective partially humanized TCR and for the 21 bases lying 5'-terminally of the human CD3ζ chain **(Figure 7)**. The chimeric constructs are humanized from the constant domain onward.

3.) Transduction of human peripheral blood lymphocytes (PBLs)

For the transduction of human peripheral T-lymphocytes, a functional derivative of the pStitch system was used (Weijtens et al., 1999). The retroviral genes necessary for the packaging are encoded by means of individual plasmids in the course of a cotransfection of the packaging cell line 293T (Soneoka et al., 1995): pHit60 codes for the *gag-pol* structure and polymerase genes from the Moloney murine leukemia virus (MoMuLV), pColt-Galv for the *env* coat proteins of the "gibbon ape leukemia virus", which is capable of binding to the Na^+/phosphate synporter pit of human cells and thus of transducing the latter. The chimeric virus particles thus possess an amphotropic pseudotype and can transduce various mammalian cells, except for mouse.

3.1) Transfection of the packaging cell line 293T

The isolated bacterial clones of the T-cell receptor genes cloned in the pStitch derivative were purified by means of plasmid preparations which promise a removal of residual endotoxins (Qiagen, product 12362), and adjusted to 1 µg/µl. The DNA was inserted transiently into the packaging cell line 293T by means of the calcium phosphate precipitation (GiboBRL-Life Technologies, product 18306-019). In this connection, in the context of the WT or modified double-chain T-cell receptors αTCR and βTCR up to 80 µg of DNA are employed:

20 µg of αTCR construct

20 µg of βTCR construct
20 µg of pColt-Galv
20 µg of pHit 60

In the case of the single-chain TCRs, 60 µg of DNA are employed. 293T was cultured in a modified DMEM medium (DMEM/H):

DMEM, 4.5% glucose (BioWhittaker)
10% of heat-inactivated FCS
2mM glutamine
1x penicillin/streptomycin
1x nonessential amino acids
25 mM HEPES

On the day before the transfection, the cells 293T were inoculated in 5ml DMEM/H at 0.9 x 10^6 cells per T25 bottle and transfection batch. 4 h before transfection, the medium was replaced by fresh DMEM-H (3ml) warmed to room temperature (RT). The transfection was carried out according to the directions of the commercial protocol (Life Technologies). 1 ml of the transfection batch was pipetted to the respective bottle with careful dropwise addition. The DNA-$Ca_3(PO_4)_2$ precipitate should be deposited on the adherent cells in finely dispersed form.

On the following morning, the medium was exchanged for fresh DMEM/H warmed to RT. 6 h later, the coculturing with the activated PBLs was carried out.

3.2) Transduction of the activated PBLs

3.2.1) Activation of periphal blood lymphocytes (PBLs)

3 days before the scheduled coculturing, ficollized PBLs were inoculated at 2 x 10^6 cells/ml in huRPMI-P in each case in 2ml of a 24-well plate (cell tissue-treated surfaces). The activation was carried out by means of the crosslinking antibody OKT-3 (Orthoclone Diagnostics) to 20 ng/ml.

huRPMI-P:
RPMI 1640 (2mM glutamine) without phosphate (Life Tec., 11877-032)
10 % human, heat-inactivated AB serum (HLA-A2.1 seropositive)
25 mM HEPES
1 x penicillin/streptomycin (Life Tec.)

The plates were incubated in an incubator at 37°C and under 5% CO_2.

3.2.2) Coculturing

For the coculturing, the activated PBLs from the respective hollows of a 24-well plate were combined and counted. Adherent monocytes were discarded. The cells are centrifuged off (1500 rpm, 5 min, RT) and taken up in a concentration of 2.5 x 10^6 cells/ml in fresh huRPMI-P and put back into the incubator. The medium was adjusted beforehand to 400 U/ml of IL-2 (Chiron) and 5 µg/ml of Polybrene (Sigma).

Each transfection batch was successively trypsinized 6h after medium change: for this, each T25 was washed with 3ml of HBSS (Life Technologies), incubated with 1 ml of trypsin-EDTA (Life Technologies) for at most 5 minutes, the dissolved cells were taken up quantitatively and added dropwise to 4ml of initially introduced huRPMI-P (RT) with stirring. The 293T cells are irradiated with 2500 rad. These cells are centrifuged off (1500 rpm, 5 min, RT) and resuspended in 4 ml of fresh, adjusted huRPMI-P, supplemented with 400U/ml of IL-2 and 5µg/ml of Polybrene. 1 ml of the adjusted PBLs was added to the batch and the batch (0.5 x 10^6 PBLs/ml) was incubated for three days in an incubator (37°C, 5% CO2).

On day 3 after coculturing, the suspended PBLs were removed and resuspended in fresh medium huRPMI-P, supplemented with IL-2 (400 U/ml), to 0.5 x 10^6 cells/

ml. 3 days later, a repeated split into fresh medium was carried out. In these 7 days, the culture was expanded maximally up to the change to T75 bottles. It was possible to employ these cells directly in an immunological staining (FACS analysis; Chap. 3.3, **Figure 9**) or in a conventional ^{51}chromium release test (Chap. 3.4, **Figures 10-15**).

3.3) Examples of the FACS analysis

The constructs described under 2.) were analyzed after retroviral transduction in the "fluorescence activated cell sorting" (FACS). For this, 0.25 x 10^6 cells were stained under saturating conditions with fluorophore-labeled antibodies: the heterologously expressed β-chain was detected using anti-vβ6-FITC (BD); the whole of the T-cells by means of the label anti-CD3-PC5 (Coulter-Beckman) **(Figure 3)**.

The negative control used was a sample transduced with the empty pStitch derivative. The wild-type muvα-mucα/muvβ-mucβ (**2.1**) and the chimeric constructs muvα-hucα/muvβ-hucβ (**2.2**) achieved transduction efficiencies in the range from 10-25% 6 days after coculturing. It was possible to reproduce the expression in a number of donors of HLA-A2 - positive T-cells. The cytosolically expressed green-fluorescing protein (eGFP, recloned from eGFP-N1 from Clontech) was used as an external standard and showed that the comparatively weak transduction efficiency as an expression of the percentage detection of successfully transduced T-cells is a methodological problem and not an intrinsic problem of the TCR transgenes.

3.3.1) Enrichment of vβ6-positive T-cells by means of magnetic sorting

In order to enrich the successfully transduced T-cells, these were sorted in a magnetic field by means of magnetically labeled vβ6 antibodies (according to the procedure of Miltenyi-Biotec). For this, a magnetically

labeled antibody was used which recognizes the rat IgG component of the vβ6-specific antibodies (Miltenyi-Biotec "Goat anti-rat IgG MicroBeads", product 485-01). It was possible to achieve enrichments of vβ6-positive $CD3^+$ - T-cells of over 90%.

3.4) Cytolytic activity of the transduced T-cells

The transduced T-cells were checked for their cytotoxic specificity in a conventional ^{51}chromium release test. In this system, target cells were radiolabeled by incorporation of ^{51}chromium. If the retrovirally modified effector cells recognized the target cell peptide-specifically, the latter were driven into apoptosis by the effector functions of the T-cell and destroyed by lysis. The extent of the chromium nuclide released makes a statement about the effectiveness of the cell recognition and lysis. The effectiveness was tested by means of a wide range of the ratio of effector cells employed to target cells (E:T). The reference used was the murine hdm2-81-88 peptide-specific T-cell clone 3, from which the T-cell receptor genes were isolated. The target cells used were:

T2: human TAP-deficient cell line which was to be loaded exogenously with any desired peptide. The specific peptide was hdm2-81-88, an irrelevant control peptide originating from the influenza matrix protein FluM1.

Saos-2/6: transfectant of the human Saos-2 cell line, which expresses and endogenously processes hdm2.

Saos2/143: transfectant of the human Saos-2 cell line, which carries the p53 mutant $Val^{143}Ala$. The p53 mutant regulates the expression of hdm2 in a positive manner

JY: EBV-transformed LCL-B cell line

UocB 1 1, EU-3: pre-B cell leukemia

IM-9: plasmacytoma

3.4.1) Peptide titration (Figures 10-12)

The cell line T2 was loaded exogenously with the hdm2-81-88 peptide in a concentration series from 1 x 10^{-6}M to 1 x 10^{-12}M. It was possible both for muvα-mucα/muvβ-mucβ (**2.1**) and muvα-hucα/muvβ-hucβ (**2.2**) to observe a peptide-specific and dose-dependent cytotoxic T-cell response. For the two constructs, at E:T = 20:1 the half-maximal lyses lay in the subnanomolar range of peptide employed. A nonfunctional single-chain T-cell receptor functioned as the negative control, which although it was expressed and could be enriched according to 3.3.1), showed no lysis activity. The positive control used was the murine hdm2-81-88 peptid-specific T-cell clone 3.

3.4.2) Recognition of malignant transformed cell lines (Figures 13-15)

It was possible for the cell lines described above to be lyzed peptide-specifically. It was possible for some of the pre-B-cell leukemias to be lyzed to 100% at high E:T's in the case of the WT chains. Just as in the case of the peptide titration, the wild-type chains (2.1) showed a higher lysis efficiency in comparison to the partially humanized, chimeric chains (2.2). A nonfunctional single-chain T-cell receptor functioned as the negative control, which although it was expressed and could be enriched according to 3.3.1), showed no lysis activity. The positive control used was the murine hdm2-81-88 peptide-specific T-cell clone 3.

SEQUENCE LISTING

<110> Stanislawski, Thomas

<120> Polypeptides of an hdm2 protein-specific, murine alpha/beta T-cell receptor, nucleic acids encoding these and their use

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Ser Gln Thr Thr Asp Ser Gly Thr Tyr Phe Cys Ala Thr Ser Ser Val
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Asn Thr Gly Asn Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys
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130 135 140

Asp Pro Arg Ser Gln Asp Ser Thr Leu Cys Leu Phe Thr Asp Phe Asp
145 150 155 160

Ser Gln Ile Asn Val Pro Lys Thr Met Glu Ser Gly Thr Phe Ile Thr
165 170 175

Asp Lys Thr Val Leu Asp Met Lys Ala Met Asp Ser Lys Ser Asn Gly
180 185 190

Ala Ile Ala Trp Ser Asn Gln Thr Ser Phe Thr Cys Gln Asp Ile Phe
195 200 205

Lys Glu Thr Asn Ala Thr Tyr Pro Ser Ser Asp Val Pro Cys Asp Ala

```
     210                 215                 220

Thr Leu Thr Glu Lys Ser Phe Glu Thr Asp Met Asn Leu Asn Phe Gln
225                 230                 235                 240

Asn Leu Ser Val Met Gly Leu Arg Ile Leu Leu Leu Lys Val Ala Gly
                245                 250                 255

Phe Asn Leu Leu Met Thr Leu Arg Leu Trp Ser Ser
            260                 265
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<210> 2
<211> 306
<212> PRT
<213> Mus musculus

<400> 2

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Met Asn Lys Trp Val Phe Cys Trp Val Thr Leu Cys Leu Leu Thr Val
  1               5                  10                  15

Glu Thr Thr His Gly Asp Gly Gly Ile Ile Thr Gln Thr Pro Lys Phe
            20                  25                  30

Leu Ile Gly Gln Glu Gly Gln Lys Leu Thr Leu Lys Cys Gln Gln Asn
        35                  40                  45

Phe Asn His Asp Thr Met Tyr Trp Tyr Arg Gln Asp Ser Gly Lys Gly
    50                  55                  60

Leu Arg Leu Ile Tyr Tyr Ser Ile Thr Glu Asn Asp Leu Gln Lys Gly
65                  70                  75                  80

Asp Leu Ser Glu Gly Tyr Asp Ala Ser Arg Glu Lys Lys Ser Ser Phe
                85                  90                  95

Ser Leu Thr Val Thr Ser Ala Gln Lys Asn Glu Met Ala Val Phe Leu
            100                 105                 110

Cys Ala Ser Gly Asp Trp Gly Tyr Glu Gln Tyr Phe Gly Pro Gly Thr
        115                 120                 125

Arg Leu Thr Val Leu Glu Asp Leu Arg Asn Val Thr Pro Pro Lys Val
    130                 135                 140

Ser Leu Phe Glu Pro Ser Lys Ala Glu Ile Ala Asn Lys Gln Lys Ala
145                 150                 155                 160

Thr Leu Val Cys Leu Ala Arg Gly Phe Phe Pro Asp His Val Glu Leu
                165                 170                 175

Ser Trp Trp Val Asn Gly Lys Glu Val His Ser Gly Val Ser Thr Asp
            180                 185                 190

Pro Gln Ala Tyr Lys Glu Ser Asn Tyr Ser Tyr Cys Leu Ser Ser Arg
        195                 200                 205

Leu Arg Val Ser Ala Thr Phe Trp His Asn Pro Arg Asn His Phe Arg
    210                 215                 220

Cys Gln Val Gln Phe His Gly Leu Ser Glu Glu Asp Lys Trp Pro Glu
225                 230                 235                 240
```

Gly Ser Pro Lys Pro Val Thr Gln Asn Ile Ser Ala Glu Ala Trp Gly
245 250 255

Arg Ala Asp Cys Gly Ile Thr Ser Ala Ser Tyr His Gln Gly Val Leu
260 265 270

Ser Ala Thr Ile Leu Tyr Glu Ile Leu Leu Gly Lys Ala Thr Leu Tyr
275 280 285

Ala Val Leu Val Ser Gly Leu Val Leu Met Ala Met Val Lys Lys Lys
290 295 300

Asn Ser
305

<210> 3
<211> 272
<212> PRT
<213> Mus musculus

<400> 3
Met Lys Arg Leu Leu Cys Ser Leu Leu Gly Leu Leu Cys Thr Gln Val
1 5 10 15

Cys Trp Val Lys Gly Gln Gln Val Gln Gln Ser Pro Ala Ser Leu Val
20 25 30

Leu Gln Glu Gly Glu Asn Ala Glu Leu Gln Cys Asn Phe Ser Ser Thr
35 40 45

Ala Thr Arg Leu Gln Trp Phe Tyr Gln Arg Pro Gly Gly Ser Leu Val
50 55 60

Ser Leu Leu Tyr Asn Pro Ser Gly Thr Lys His Thr Gly Arg Leu Thr
65 70 75 80

Ser Thr Thr Val Thr Lys Glu Arg Arg Ser Ser Leu His Ile Ser Ser
85 90 95

Ser Gln Thr Thr Asp Ser Gly Thr Tyr Phe Cys Ala Thr Ser Ser Val
100 105 110

Asn Thr Gly Asn Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys
115 120 125

Val Ile Ala His Ile Gln Asn Pro Glu Pro Ala Val Tyr Gln Leu Arg
130 135 140

Asp Ser Lys Ser Ser Asp Lys Ser Val Cys Leu Phe Thr Asp Phe Asp
145 150 155 160

Ser Gln Thr Asn Val Ser Gln Ser Lys Asp Ser Asp Val Tyr Ile Thr
165 170 175

Asp Lys Thr Val Leu Asp Met Arg Ser Met Asp Phe Lys Ser Asn Ser
180 185 190

Ala Val Ala Trp Ser Asn Lys Ser Asp Phe Ala Cys Ala Asn Ala Phe
195 200 205

```
Asn Asn Ser Ile Ile Pro Glu Asp Thr Phe Phe Pro Ser Pro Glu Ser
    210                 215                 220

Ser Cys Asp Val Lys Leu Val Glu Lys Ser Phe Glu Thr Asp Thr Asn
225                 230                 235                 240

Leu Asn Phe Gln Asn Leu Ser Val Ile Gly Phe Arg Ile Leu Leu Leu
                245                 250                 255

Lys Val Ala Gly Phe Asn Leu Leu Met Thr Leu Arg Leu Trp Ser Ser
            260                 265                 270
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<2l0> 4
<211> 310
<212> PRT
<213> Mus musculus

<400> 4

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Met Asn Lys Trp Val Phe Cys Trp Val Thr Leu Cys Leu Leu Thr Val
  1               5                  10                  15

Glu Thr Thr His Gly Asp Gly Gly Ile Ile Thr Gln Thr Pro Lys Phe
            20                  25                  30

Leu Ile Gly Gln Glu Gly Gln Lys Leu Thr Leu Lys Cys Gln Gln Asn
        35                  40                  45

Phe Asn His Asp Thr Met Tyr Trp Tyr Arg Gln Asp Ser Gly Lys Gly
    50                  55                  60

Leu Arg Leu Ile Tyr Tyr Ser Ile Thr Glu Asn Asp Leu Gln Lys Gly
65                  70                  75                  80

Asp Leu Ser Glu Gly Tyr Asp Ala Ser Arg Glu Lys Lys Ser Ser Phe
                85                  90                  95

Ser Leu Thr Val Thr Ser Ala Gln Lys Asn Glu Met Ala Val Phe Leu
            100                 105                 110

Cys Ala Ser Gly Asp Trp Gly Tyr Glu Gln Tyr Phe Gly Pro Gly Thr
        115                 120                 125

Arg Leu Thr Val Leu Glu Asp Leu Asn Lys Val Phe Pro Pro Glu Val
    130                 135                 140

Ala Val Phe Glu Pro Ser Glu Ala Glu Ile Ser His Thr Gln Lys Ala
145                 150                 155                 160

Thr Leu Val Cys Leu Ala Thr Gly Phe Phe Pro Asp His Val Glu Leu
                165                 170                 175

Ser Trp Trp Val Asn Gly Lys Glu Val His Ser Gly Val Ser Thr Asp
            180                 185                 190

Pro Gln Pro Leu Lys Glu Gln Pro Ala Leu Asn Asp Ser Arg Tyr Cys
        195                 200                 205

Leu Ser Ser Arg Leu Arg Val Ser Ala Thr Phe Trp Gln Asn Pro Arg
```

210 215 220

Asn His Phe Arg Cys Gln Val Gln Phe Tyr Gly Leu Ser Glu Asn Asp
225 230 235 240

Glu Trp Thr Gln Asp Arg Ala Lys Pro Val Thr Gln Ile Val Ser Ala
245 250 255

Glu Ala Trp Gly Arg Ala Asp Cys Gly Phe Thr Ser Val Ser Tyr Gln
260 265 270

Gln Gly Val Leu Ser Ala Thr Ile Leu Tyr Glu Ile Leu Leu Gly Lys
275 280 285

Ala Thr Leu Tyr Ala Val Leu Val Ser Ala Leu Val Leu Met Ala Met
290 295 300

Val Lys Arg Lys Asp Phe
305 310

<210> 5
<211> 430
<212> PRT
<213> Mus musculus

<400> 5
Met Lys Arg Leu Leu Cys Ser Leu Leu Gly Leu Leu Cys Thr Gln Val
1 5 10 15

Cys Trp Val Lys Gly Gln Gln Val Gln Gln Ser Pro Ala Ser Leu Val
20 25 30

Leu Gln Glu Gly Glu Asn Ala Glu Leu Gln Cys Asn Phe Ser Ser Thr
35 40 45

Ala Thr Arg Leu Gln Trp Phe Tyr Gln Arg Pro Gly Gly Ser Leu Val
50 55 60

Ser Leu Leu Tyr Asn Pro Ser Gly Thr Lys His Thr Gly Arg Leu Thr
65 70 75 80

Ser Thr Thr Val Thr Lys Glu Arg Arg Ser Ser Leu His Ile Ser Ser
85 90 95

Ser Gln Thr Thr Asp Ser Gly Thr Tyr Phe Cys Ala Thr Ser Ser Val
100 105 110

Asn Thr Gly Asn Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys
115 120 125

Val Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
130 135 140

Arg Asp Gly Gly Ile Ile Thr Gln Thr Pro Lys Phe Leu Ile Gly Gln
145 150 155 160

Glu Gly Gln Lys Leu Thr Leu Lys Cys Gln Gln Asn Phe Asn His Asp
165 170 175

Thr Met Tyr Trp Tyr Arg Gln Asp Ser Gly Lys Gly Leu Arg Leu Ile
180 185 190

```
Tyr Tyr Ser Ile Thr Glu Asn Asp Leu Gln Lys Gly Asp Leu Ser Glu
        195                 200                 205

Gly Tyr Asp Ala Ser Arg Glu Lys Lys Ser Ser Phe Ser Leu Thr Val
    210                 215                 220

Thr Ser Ala Gln Lys Asn Glu Met Ala Val Phe Leu Cys Ala Ser Gly
225                 230                 235                 240

Asp Trp Gly Tyr Glu Gln Tyr Phe Gly Pro Gly Thr Arg Leu Thr Val
                245                 250                 255

Leu Glu Asp Leu Arg Asn Val Thr Pro Pro Lys Val Ser Leu Phe Glu
            260                 265                 270

Pro Ser Lys Ala Glu Ile Ala Asn Lys Gln Lys Ala Thr Leu Val Cys
        275                 280                 285

Leu Ala Arg Gly Phe Phe Pro Asp His Val Glu Leu Ser Trp Trp Val
    290                 295                 300

Asn Gly Lys Glu Val His Ser Gly Val Ser Thr Asp Pro Gln Ala Tyr
305                 310                 315                 320

Lys Glu Ser Asn Tyr Ser Tyr Cys Leu Ser Ser Arg Leu Arg Val Ser
                325                 330                 335

Ala Thr Phe Trp His Asn Pro Arg Asn His Phe Arg Cys Gln Val Gln
            340                 345                 350

Phe His Gly Leu Ser Glu Glu Asp Lys Trp Pro Glu Gly Ser Pro Lys
        355                 360                 365

Pro Val Thr Gln Asn Ile Ser Ala Glu Ala Trp Gly Arg Ala Asp Cys
    370                 375                 380

Gly Ile Thr Ser Ala Ser Tyr His Gln Gly Val Leu Ser Ala Thr Ile
385                 390                 395                 400

Leu Tyr Glu Ile Leu Leu Gly Lys Ala Thr Leu Tyr Ala Val Leu Val
                405                 410                 415

Ser Gly Leu Val Leu Met Ala Met Val Lys Lys Lys Asn Ser
            420                 425                 430
```

<210> 6
<211> 392
<212> PRT
<213> Mus musculus

<220>
<223> sequence is partially humanized (chimeric)

<400> 6

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Met Asn Lys Trp Val Phe Cys Trp Val Thr Leu Cys Leu Leu Thr Val
  1               5                  10                  15

Glu Thr Thr His Gly Asp Gly Gly Ile Ile Thr Gln Thr Pro Lys Phe
             20                  25                  30
```

Leu Ile Gly Gln Glu Gly Gln Lys Leu Thr Leu Lys Cys Gln Gln Asn
35 40 45

Phe Asn His Asp Thr Met Tyr Trp Tyr Arg Gln Asp Ser Gly Lys Gly
50 55 60

Leu Arg Leu Ile Tyr Tyr Ser Ile Thr Glu Asn Asp Leu Gln Lys Gly
65 70 75 80

Asp Leu Ser Glu Gly Tyr Asp Ala Ser Arg Glu Lys Lys Ser Ser Phe
85 90 95

Ser Leu Thr Val Thr Ser Ala Gln Lys Asn Glu Met Ala Val Phe Leu
100 105 110

Cys Ala Ser Gly Asp Trp Gly Tyr Glu Gln Tyr Phe Gly Pro Gly Thr
115 120 125

Arg Leu Thr Val Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly
130 135 140

Gly Gly Ser Val Gln Gln Ser Pro Ala Ser Leu Val Leu Gln Glu Gly
145 150 155 160

Glu Asn Ala Glu Leu Gln Cys Asn Phe Ser Ser Thr Ala Thr Arg Leu
165 170 175

Gln Trp Phe Tyr Gln Arg Pro Gly Gly Ser Leu Val Ser Leu Leu Tyr
180 185 190

Asn Pro Ser Gly Thr Lys His Thr Gly Arg Leu Thr Ser Thr Thr Val
195 200 205

Thr Lys Glu Arg Arg Ser Ser Leu His Ile Ser Ser Ser Gln Thr Thr
210 215 220

Asp Ser Gly Thr Tyr Phe Cys Ala Thr Ser Ser Val Asn Thr Gly Asn
225 230 235 240

Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys Val Ile Ala His
245 250 255

Ile Gln Asn Pro Glu Pro Ala Val Tyr Gln Leu Lys Asp Pro Arg Ser
260 265 270

Gln Asp Ser Thr Leu Cys Leu Phe Thr Asp Phe Asp Ser Gln Ile Asn
275 280 285

Val Pro Lys Thr Met Glu Ser Gly Thr Phe Ile Thr Asp Lys Thr Val
290 295 300

Leu Asp Met Lys Ala Met Asp Ser Lys Ser Asn Gly Ala Ile Ala Trp
305 310 315 320

Ser Asn Gln Thr Ser Phe Thr Cys Gln Asp Ile Phe Lys Glu Thr Asn
325 330 335

Ala Thr Tyr Pro Ser Ser Asp Val Pro Cys Asp Ala Thr Leu Thr Glu
340 345 350

Lys Ser Phe Glu Thr Asp Met Asn Leu Asn Phe Gln Asn Leu Ser Val
355 360 365

Met Gly Leu Arg Ile Leu Leu Leu Lys Val Ala Gly Phe Asn Leu Leu
370 375 380

Met Thr Leu Arg Leu Trp Ser Ser
385 390

<210> 7
<211> 357
<212> PRT
<213> Mus musculus

<400> 7
Met Glu Arg Leu Leu Cys Ser Leu Leu Gly Leu Leu Cys Thr Gln Val
1 5 10 15

Cys Trp Val Lys Gly Gln Gln Val Gln Gln Ser Pro Ala Ser Leu Val
20 25 30

Leu Gln Glu Gly Glu Asn Ala Glu Leu Gln Cys Asn Phe Ser Ser Thr
35 40 45

Ala Thr Arg Leu Gln Trp Phe Tyr Gln Arg Pro Gly Gly Ser Leu Val
50 55 60

Ser Leu Leu Tyr Asn Pro Ser Gly Thr Lys His Thr Gly Arg Leu Thr
65 70 75 80

Ser Thr Thr Val Thr Lys Glu Arg Arg Ser Ser Leu His Ile Ser Ser
85 90 95

Ser Gln Thr Thr Asp Ser Gly Thr Tyr Phe Cys Ala Thr Ser Ser Val
100 105 110

Asn Thr Gly Asn Tyr Lys Tyr Val Phe Gly Ala Gly Thr Arg Leu Lys
115 120 125

Val Ile Ala His Ile Gln Asn Pro Glu Pro Ala Val Tyr Gln Leu Arg
130 135 140

Asp Ser Lys Ser Ser Asp Lys Ser Val Cys Leu Phe Thr Asp Phe Asp
145 150 155 160

Ser Gln Thr Asn Val Ser Gln Ser Lys Asp Ser Asp Val Tyr Ile Thr
165 170 175

Asp Lys Thr Val Leu Asp Met Arg Ser Met Asp Phe Lys Ser Asn Ser
180 185 190

Ala Val Ala Trp Ser Asn Lys Ser Asp Phe Ala Cys Ala Asn Ala Phe
195 200 205

Asn Asn Ser Ile Ile Pro Glu Asp Thr Phe Phe Pro Ser Pro Glu Ser
210 215 220

Ser Cys Tyr Leu Leu Asp Gly Ile Leu Phe Ile Tyr Gly Val Ile Leu
225 230 235 240

Thr Ala Leu Phe Leu Arg Val Lys Phe Ser Arg Ser Ala Asp Ala Pro
245 250 255

Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr Asn Glu Leu Asn Leu Gly

260 265 270

Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys Arg Arg Gly Arg Asp Pro
275 280 285

Glu Met Gly Gly Lys Pro Arg Arg Lys Asn Pro Gln Glu Gly Leu Tyr
290 295 300

Asn Glu Leu Gln Lys Asp Lys Met Ala Glu Ala Tyr Ser Glu Ile Gly
305 310 315 320

Met Lys Gly Glu Arg Arg Arg Gly Lys Gly His Asp Gly Leu Tyr Gln
325 330 335

Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr Asp Ala Leu His Met Gln
340 345 350

Ala Leu Pro Pro Arg
355

<210> 8
<211> 395
<212> PRT
<213> Mus musculus

<400> 8
Met Asp Lys Trp Val Phe Cys Trp Val Thr Leu Cys Leu Leu Thr Val
1 5 10 15

Glu Thr Thr His Gly Asp Gly Gly Ile Ile Thr Gln Thr Pro Lys Phe
20 25 30

Leu Ile Gly Gln Glu Gly Gln Lys Leu Thr Leu Lys Cys Gln Gln Asn
35 40 45

Phe Asn His Asp Thr Met Tyr Trp Tyr Arg Gln Asp Ser Gly Lys Gly
50 55 60

Leu Arg Leu Ile Tyr Tyr Ser Ile Thr Glu Asn Asp Leu Gln Lys Gly
65 70 75 80

Asp Leu Ser Glu Gly Tyr Asp Ala Ser Arg Glu Lys Lys Ser Ser Phe
85 90 95

Ser Leu Thr Val Thr Ser Ala Gln Lys Asn Glu Met Ala Val Phe Leu
100 105 110

Cys Ala Ser Gly Asp Trp Gly Tyr Glu Gln Tyr Phe Gly Pro Gly Thr
115 120 125

Arg Leu Thr Val Leu Glu Asp Leu Asn Lys Val Phe Pro Pro Glu Val
130 135 140

Ala Val Phe Glu Pro Ser Glu Ala Glu Ile Ser His Thr Gln Lys Ala
145 150 155 160

Thr Leu Val Cys Leu Ala Thr Gly Phe Phe Pro Asp His Val Glu Leu
165 170 175

Ser Trp Trp Val Asn Gly Lys Glu Val His Ser Gly Val Ser Thr Asp
180 185 190

Pro Gln Pro Leu Lys Glu Gln Pro Ala Leu Asn Asp Ser Arg Tyr Cys
195 200 205

Leu Ser Ser Arg Leu Arg Val Ser Ala Thr Phe Trp Gln Asn Pro Arg
210 215 220

Asn His Phe Arg Cys Gln Val Gln Phe Tyr Gly Leu Ser Glu Asn Asp
225 230 235 240

Glu Trp Thr Gln Asp Arg Ala Lys Pro Val Thr Gln Ile Val Ser Ala
245 250 255

Glu Ala Trp Gly Arg Ala Asp Cys Tyr Leu Leu Asp Gly Ile Leu Phe
260 265 270

Ile Tyr Gly Val Ile Leu Thr Ala Leu Phe Leu Arg Val Lys Phe Ser
275 280 285

Arg Ser Ala Asp Ala Pro Ala Tyr Gln Gln Gly Gln Asn Gln Leu Tyr
290 295 300

Asn Glu Leu Asn Leu Gly Arg Arg Glu Glu Tyr Asp Val Leu Asp Lys
305 310 315 320

Arg Arg Gly Arg Asp Pro Glu Met Gly Gly Lys Pro Arg Arg Lys Asn
325 330 335

Pro Gln Glu Gly Leu Tyr Asn Glu Leu Gln Lys Asp Lys Met Ala Glu
340 345 350

Ala Tyr Ser Glu Ile Gly Met Lys Gly Glu Arg Arg Arg Gly Lys Gly
355 360 365

His Asp Gly Leu Tyr Gln Gly Leu Ser Thr Ala Thr Lys Asp Thr Tyr
370 375 380

Asp Ala Leu His Met Gln Ala Leu Pro Pro Arg
385 390 395

<210> 9
<211> 19
<212> DNA
<213> Mus musculus

<400> 9
tyatccarac wccaarvta 19

<210> 10
<211> 19
<212> DNA
<213> Mus musculus

<400> 10
ggatctcata gaggatggt 19

<210> 11
<211> 28
<212> DNA

<213> Mus musculus

<400> 11
ggaattcgty atccaracwc caarytaa 28

<210> 12
<211> 31
<212> DNA
<213> Mus musculus

<400> 12
aggcgcgcct ggccacttgt cctcctctga a 31

<210> 13
<211> 27
<212> DNA
<213> Mus musculus

<400> 13
ggaattccga ggatctgaga aatgtga 27

<210> 14
<211> 34
<212> DNA
<213> Mus musculus

<400> 14
aggcgcgcct tcaggaatty tttytyttga ccat 34

<210> 15
<211> 34
<212> DNA
<213> Mus musculus

<400> 15
aggcgcgcct tcaactggac cacagcctca gcgt 34

<210> 16
<211> 19
<212> DNA
<213> Mus musculus

<400> 16
aggttcatat ctgtttcaa 19

<210> 17
<211> 28
<212> DNA
<213> Mus musculus

<400> 17
aggcgcgcct ggcgttggtc tctttgaa 28

<210> 18
<211> 32
<212> DNA

<213> Mus musculus

<400> 18
ggaattcccc agaacccaga acctgctgtg ta 32

<210> 19
<211> 31
<212> DNA
<213> Mus musculus

<400> 19
ccatgaccat ggaaaggctg ctgtgctctc t 31

<210> 20
<211> 20
<212> DNA
<213> Mus musculus

<400> 20
gaaaggctgc tgtgctctct 20

<210> 21
<211> 35
<212> DNA
<213> Mus musculus

<400> 21
ccatgaccat ggacaagtgg gttttctgct gggta 35

<210> 22
<211> 24
<212> DNA
<213> Mus musculus

<400> 22
gacaagtggg ttttctgctg ggta 24

<210> 23
<211> 34
<212> DNA
<213> Mus musculus

<400> 23
cgggatccgc tcaactggac cacagcctca gcgt 34

<210> 24
<211> 34
<212> DNA
<213> Mus musculus

<400> 24
cgggatccgc tcaggaattt tttttcttga ccat 34

<210> 25
<211> 20
<212> DNA

<213> Mus musculus

<400> 25
atatccagaa ccctgaccct 20

<210> 26
<211> 21
<212> DNA
<213> Mus musculus

<400> 26
gggagcacag gctgtcttac a 21

<210> 27
<211> 40
<212> DNA
<213> Mus musculus

<400> 27
cagaacccag aacctgccgt gtaccagctg agagactcta 40

<210> 28
<211> 37
<212> DNA
<213> Mus musculus

<400> 28
cgggatcctc agctggacca cagccgcagc gtcatga 37

<210> 29
<211> 22
<212> DNA
<213> Mus musculus

<400> 29
tagaggacct gaacaaggtg tt 22

<210> 30
<211> 25
<212> DNA
<213> Mus musculus

<400> 30
aaccatgacg ggttagaagc tccta 25

<210> 31
<211> 22
<212> DNA
<213> Mus musculus

<400> 31
tgaacaaggt gttcccaccc ga 22

<210> 32
<211> 40
<212> DNA

<213> Mus musculus

<400> 32
cgggatcctc agaaatcctt tctcttgacc atggccatca 40

<210> 33
<211> 20
<212> DNA
<213> Mus musculus

<400> 33
taatacgact cactataggg 20

<210> 34
<211> 18
<212> DNA
<213> Mus musculus

<400> 34
tagaaggcac agtcgagg 18

<210> 35
<211> 29
<212> DNA
<213> Mus musculus

<400> 35
cgggatcctt agcgaggggg cagggcctg 29

<210> 36
<211> 42
<212> DNA
<213> Mus musculus

<400> 36
gaggattcca tccagcaggt aacaggaact ttctgggctg gg 42

<210> 37
<211> 42
<212> DNA
<213> Mus musculus

<400> 37
gaggattcca tccagcaggt aacagtctgc tctaccccag ge 42

<210> 38
<211> 807
<212> DNA
<213> Mus musculus

<400> 38
atgaaaaggc tgctgtgctc tctgctgggg cttctgtgca cacaggtttg ctgggtgaaa 60
ggacagcaag tgcagcagag tcctgcatcc ttggttctgc aggaggggga gaacgcagag 120
ctgcagtgta acttttcctc cacagcaacc cggctgcagt ggttttacca acgtcctggg 180
ggaagcctcg tcagcctgtt gtacaatcct tctgggacaa agcacactgg aagactgaca 240
tccaccacag tcactaaaga acgtcgcagc tctttgcaca tttcctcctc ccagacaaca 300
gactcaggca cttatttctg tgctacgtct tccgttaata caggaaacta caaatacgtc 360

tttggagcag gtaccagact gaaggttata gcacacatcc agaacccaga acctgctgtg 420
taccagttaa aagatcctcg gtctcaggac agcaccctct gcctgttcac cgactttgac 480
tcccaaatca atgtgccgaa aaccatggaa tctggaacgt tcatcactga caaaactgtg 540
ctggacatga aagctatgga ttccaagagc aatggggcca ttgcctggag caaccagaca 600
agcttcacct gccaagatat cttcaaagag accaacgcca cctaccccag ttcagacgtt 660
ccctgtgatg ccacgttgac tgagaaaagc tttgaaacag atatgaacct aaactttcaa 720
aacctgtcag ttatgggact ccgaatcctc ctgctgaaag tagccggatt taacctgctc 780
atgacgctga ggctgtggtc cagttga 807

<210> 39
<211> 921
<212> DNA
<213> Mus musculus

<400> 39
atgaacaagt gggttttctg ctgggtaacc ctttgtctcc ttactgtaga gaccacacat 60
ggtgatggtg gcatcattac tcagacaccc aaattcctga ttggtcagga agggcaaaaa 120
ctgaccttga aatgtcaaca gaatttcaat catgatacaa tgtactggta ccgacaggat 180
tcagggaaag gattgagact gatctactat tcaataactg aaaacgatct tcaaaaaggc 240
gatctatctg aaggctatga tgcgtctcga gagaagaagt catcttttc tctcactgtg 300
acatctgccc agaagaacga gatggccgtt tttctctgtg ccagcgggga ctgggggtat 360
gaacagtact tcggtccccg caccaggctc acggttttag aggatctgag aaatgtgact 420
ccacccaagg tctccttgtt tgagccatca aaagcagaga ttgcaaacaa acaaaaggct 480
accctcgtgt gcttggccag gggcttcttc cctgaccacg tggagctgag ctggtgggtg 540
aatggcaagg aggtccacag tggggtcagc acggaccctc aggcctacaa gcagagcaat 600
tatagctact gcctgagcag ccgcctgagg gtctctgcta ccttctggca caatcctcga 660
aaccactttc gctgccaagt gcagttccat gggctttcag aggaggacaa gtggccagag 720
ggctcaccca aacctgtcac acagaacatc agtgcagagg cctggggccg agcagactgt 780
ggaatcactt cagcatccta tcatcagggg gttctgtctg caaccatcct ctatgagatc 840
ctactgggga aggccaccct atatgctgtg ctggtcagtg gcctggtgct gatggccatg 900
gtcaagaaaa aaaattcctg a 921

<210> 40
<211> 819
<212> DNA
<213> Mus musculus

<220>
<223> sequence is partially humanized (chimeric)

<400> 40
atgaaaaggc tgctgtgctc tctgctgggg cttctgtgca cacaggtttg ctgggtgaaa 60
ggacagcaag tgcagcagag tcctgcatcc ttggttctgc aggaggggga gaacgcagag 120
ctgcagtgta acttttcctc cacagcaacc cggctgcagt ggttttacca acgtcctggg 180
ggaagcctcg tcagcctgtt gtacaatcct tctgggacaa agcacactgg aagactgaca 240
tccaccacag tcactaaaga acgtcgcagc tctttgcaca tttcctcctc ccagacaaca 300
gactcaggca cttatttctg tgctacgtct tccgttaata caggaaacta caaatacgtc 360
tttggagcag gtaccagact gaaggttata gcacacatcc agaacccaga acctgccgtg 420
taccagctga gagactctaa atccagtgac aagtctgtct gcctattcac cgattttgat 480
tctcaaacaa atgtgtcaca aagtaaggat tctgatgtgt atatcacaga caaaactgtg 540
ctagacatga ggtctatgga cttcaagagc aacagtgctg tggcctggag caacaaatct 600
gactttgcat gtgcaaacgc cttcaacaac agcattattc cagaagacac cttcttcccc 660
agcccagaaa gttcctgtga tgtcaagctg gtcgagaaaa gctttgaaac agatacgaac 720
ctaaactttc aaaacctgtc agtgattggg ttccgaatcc tcctcctgaa agtggccggg 780
tttaatctgc tcatgacgct gcggctgtgg tccagctga 819

<210> 41
<211> 933
<212> DNA
<213> Mus musculus

<220>
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Patent claims

1. A polypeptide of a murine α/β T-cell receptor mediating an hdm2 protein-specific T-cell response according to SEQ ID No. 1 or SEQ ID No. 2 or functional variants or parts thereof or nucleic acids encoding this, functional variants or parts thereof.

2. A fusion protein, comprising a polypeptide as claimed in claim 1 or functional variants or parts thereof or nucleic acids encoding this, functional variants or parts thereof.

3. The fusion protein as claimed in claim 2, characterized in that it comprises the ζ-region of CD3 and/or CD8, CD 16 or parts thereof, in particular the ζ-region of human CD3 and/or CD8, CD16 or parts thereof.

4. The fusion protein as claimed in claim 2 or 3, furthermore comprising a flexible linker, in particular a linker of the amino acid sequence $(GGGGS)_3$.

5. The fusion protein as claimed in claim 2, characterized in that it is a chimeric, at least partially humanized fusion protein, in particular according to SEQ ID No. 3 or SEQ ID No. 4.

6. The fusion protein as claimed in one of claims 2 to 5, characterized in that it is a single-chain T-cell receptor, in particular according to SEQ ID No. 5 or SEQ ID No. 6.

7. The fusion protein as claimed in one of claims 2 to 4, characterized in that it is a double-chain

T-cell receptor, in particular according to SEQ ID No. 7 or SEQ ID No. 8.

8. An α- or β- chain of a T-cell receptor, which comprises the antigen recognition sequence of an antibody specific for the amino acids 81-88 of the protein hdm2 or of a complex of hdm2 81-88 and HLA-A2.

9. The polypeptide as claimed in one of claims 1 to 8, characterized in that the polypeptide has been synthetically prepared.

10. The nucleic acid as claimed in claim 1 or 2, characterized in that it is a DNA or RNA, preferably a DNA, in particular a double-stranded DNA having a length of at least 8 nucleotides, preferably having at least 18 nucleotides, in particular having at least 24 nucleotides.

11. The nucleic acid as claimed in one of claims 1, 2 or 10, characterized in that the sequence of the nucleic acid contains at least one intron and/or one polyA sequence.

12. The nucleic acid as claimed in one of claims 1, 2, 10 or 11 in the form of its complementary "antisense" sequence.

13. The nucleic acid as claimed in one of claims 1, 2 or 10 to 12, characterized in that the nucleic acid has been synthetically prepared.

14. A vector, preferably in the form of a plasmid, shuttle vector, phagemid, cosmid, expression vector, retroviral vector, adenoviral vector or particle and/or vector active in gene therapy, comprising a nucleic acid as claimed in one of claims 1, 2 or 10 to 13.

15. A host cell, transfected with a vector or infected or transduced with a particle as claimed in claim 14.

16. The host cell as claimed in claim 15, characterized in that it is a T-cell or a T-cell precursor cell or a stem cell.

17. The host cell as claimed in claim 15 or 16, characterized in that a polypeptide or fusion protein according to one of claims 1 to 9 is expressed on its surface.

18. A process for the identification of hdm2 protein-specific antigens, characterized in that hdm2-presenting tumor cells or fractions thereof are combined with a host cell as claimed in claim 17 under conditions in which the tumor cells or fractions thereof are only lyzed if the tumor presents the hdm2 protein-specific antigen for which the expressed polypeptide or fusion protein is specific.

19. A process for the preparation of an antibody, preferably a polyclonal or monoclonal antibody, directed against a polypeptide, fusion protein or a nucleic acid as claimed in one of claims 1 to 13 for the diagnosis, treatment and/or monitoring of the treatment of diseases associated with hdm2 protein and/or for the identification of pharmacologically active substances, characterized in that an antibody-producing organism is immunized with a polypeptide or functional equivalents thereof or parts thereof having at least 6 amino acids, preferably having at least 8 amino acids, in particular having at least 12 amino acids as claimed in one of claims 1 to 9 or is vaccinated with nucleic acids encoding this.

20. An antibody, prepared as claimed in claim 19, characterized in that it is directed against a polypeptide as claimed in one of claims 1 to 9.

21. A recombinant antibody, characterized in that it comprises the antigen recognition sequence of the α- or β-chain of a T-cell receptor specific for the amino acids 81-88 of the protein hdm2 or of a complex of hdm 2 81-88 and HLA-A2.

22. A process for the production of a medicament for the treatment of diseases associated with hdm2 protein, characterized in that at least one nucleic acid, at least one polypeptide, at least one host cell or at least one antibody as claimed in one of the aforementioned claims is combined, together with suitable additives and excipients.

23. A medicament for the treatment of diseases associated with hdm2 protein, characterized in that it contains at least one nucleic acid, at least one polypeptide, at least one host cell or at least one antibody as claimed in one of the aforementioned claims, if appropriate together with suitable additives and excipients.

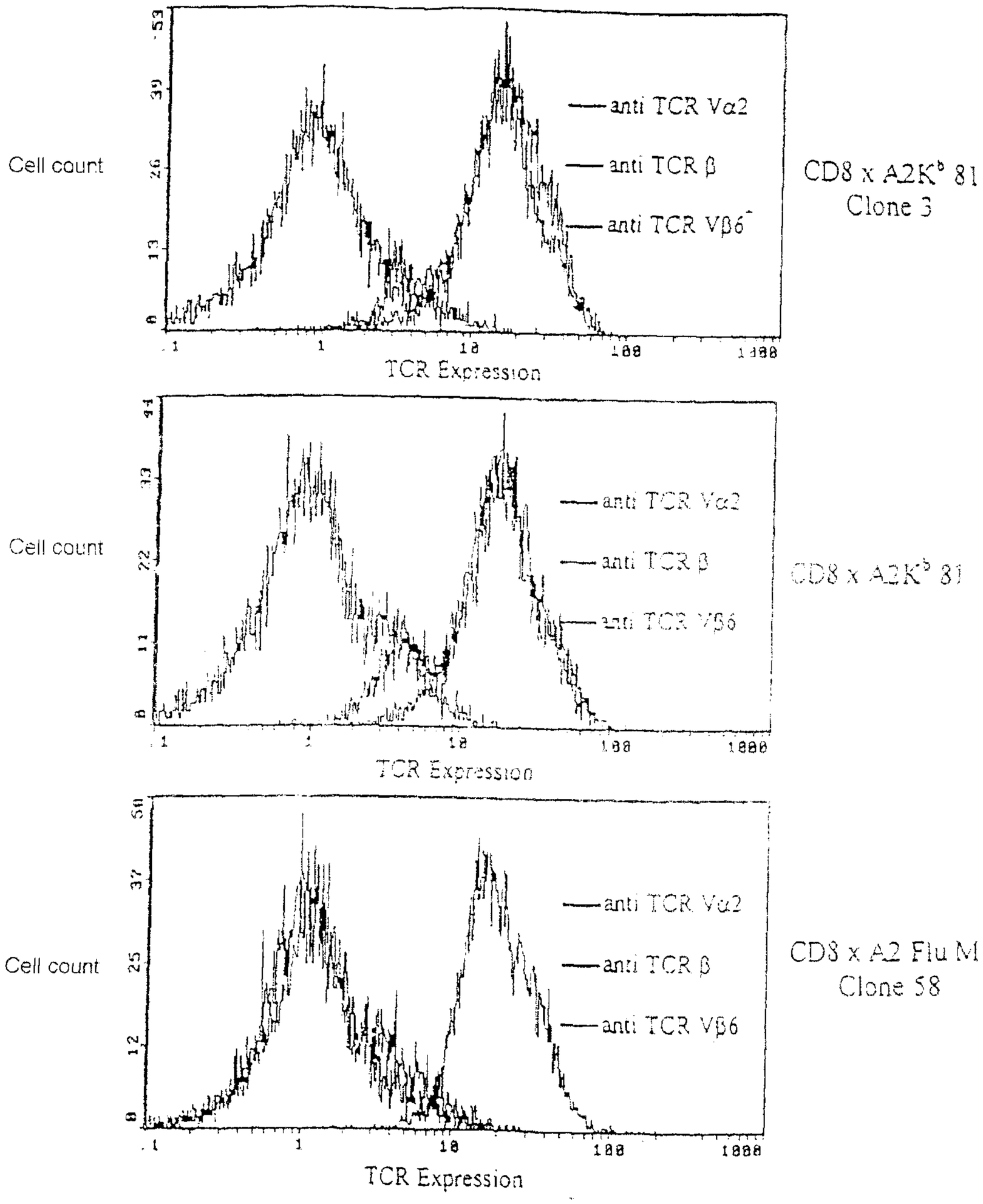
24. The use of a medicament as claimed in claim 23 for the treatment of diseases associated with hdm2 protein.

25. A process for the production of a test for the discovery of functional interactors in connection with diseases associated with hdm2 protein, characterized in that at least one nucleic acid, at least one polypeptide or at least one antibody as claimed in one of the aforementioned claims is combined, together with suitable additives and excipients.

26. A test for the identification of functional interactors in connection with diseases associated with hdm2 protein, characterized in that it contains at least one nucleic acid, at least one polypeptide or at least one antibody as claimed in one of the aforementioned claims, if appropriate together with suitable additives and excipients.

Smart & Biggar
Ottawa, Canada
Patent Agents

Figure 1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anti TCR β
anti TCR Vβ6
CD8 x A2K^b 81
Clone 3
TCR Expression
Cell count
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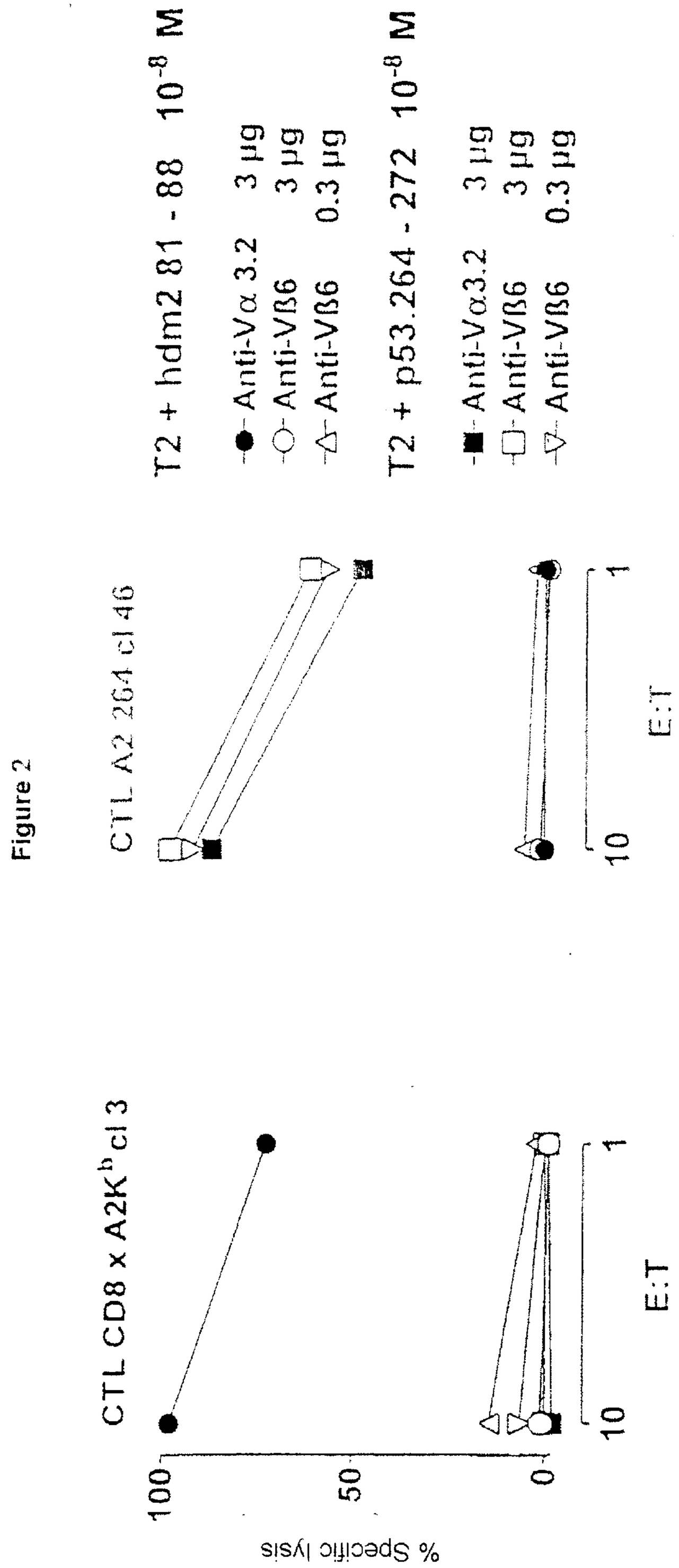
TCR Expression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CTL CD8 x A2K^b cl 3
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Anti-Vα 3.2 3 µg
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T2 + p53.264 - 272 10^{-8} M
Anti-Vα3.2 3 µg
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50
0
% Specific lysis
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10
E:T

Figure 2

Figure 3

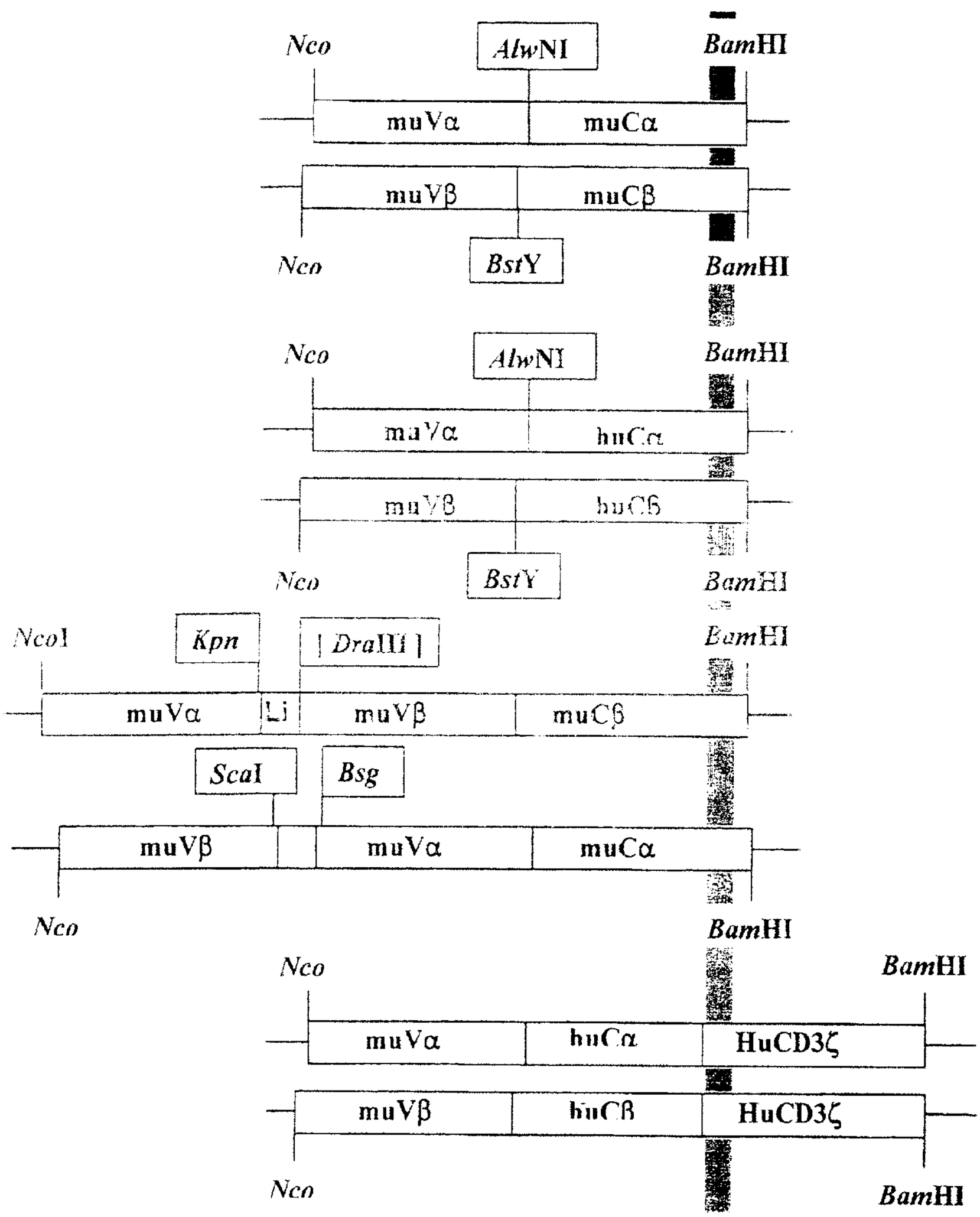
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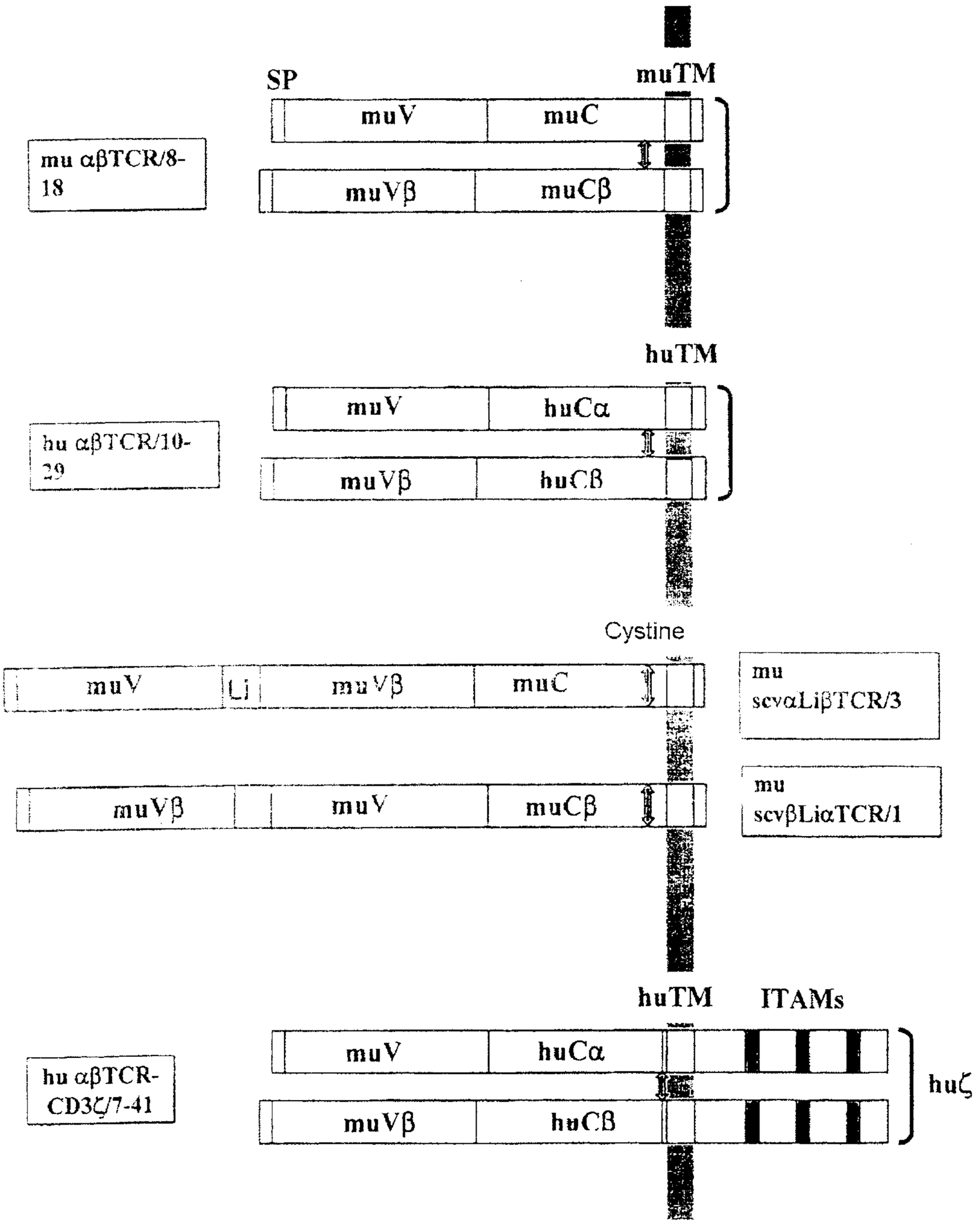
Figure 4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huζ
hu αβTCR-CD3ζ/7-41

Figure 5

Polylinker KpnI-Li(GlyGlyGlyGlySer)$_3$-DraIII-KpnI

			Gly			Thr			Arg			Leu			Lys			Val			*Gly*			*Gly*			*Gly*		
C	G	G	G	G	T	A	C	C	A	G	A	C	T	G	A	A	G	G	T	T	G	G	C	G	G	C	G	G	A

KpnI

Gly			*Ser*			*Gly*			*Gly*			*Gly*			*Gly*			*Ser*			*Gly*			*Gly*			*Gly*		
G	G	C	A	G	C	G	G	C	G	G	C	G	G	A	G	G	C	A	G	C	G	G	C	G	G	C	G	G	A

Gly			*Ser*			Gln			Gly																				
G	G	C	T	C	A	C	A	G	G	G	T	G	A	T	C	C	G	G	G	T	A	C	C	C	C	G			

Dra III **Kpn I**

Figure 6

Polylinker SeaI-Li(GlyGlyGlyGlySer)$_3$-BsgI-SeaI

															Glu			Gln			Tyr			Phe			Gly		
															G	A	A	C	A	G	T	A	C	T	T	C	G	G	T

Sca I

Pro			Gly			Thr			Arg			Leu			Thr			Val			*Gly*			*Gly*			*Gly*		
C	C	C	G	G	C	A	C	C	A	G	G	C	T	C	A	C	G	G	T	T	G	G	C	G	G	C	G	G	A

Gly			*Ser*			*Gly*			*Gly*			*Gly*			*Gly*			*Ser*			*Gly*			*Gly*			*Gly*		
G	G	C	A	G	C	G	G	C	G	G	C	G	G	A	G	G	C	A	G	C	G	G	C	G	G	C	G	G	A

Gly			*Ser*			Val			Gln			Gln			Ser			Pro			Ala			Ser			Leu		
G	G	C	A	G	C	G	T	G	C	A	G	C	A	G	A	G	T	C	C	T	G	C	A	T	C	C	T	T	G

BsgI

A	G	T	A	C	T	T	C	G	G																				

Sca I

Figure 7

Fusion of muvahuccαTCR (hdm2 81-88) with huCD3ζ

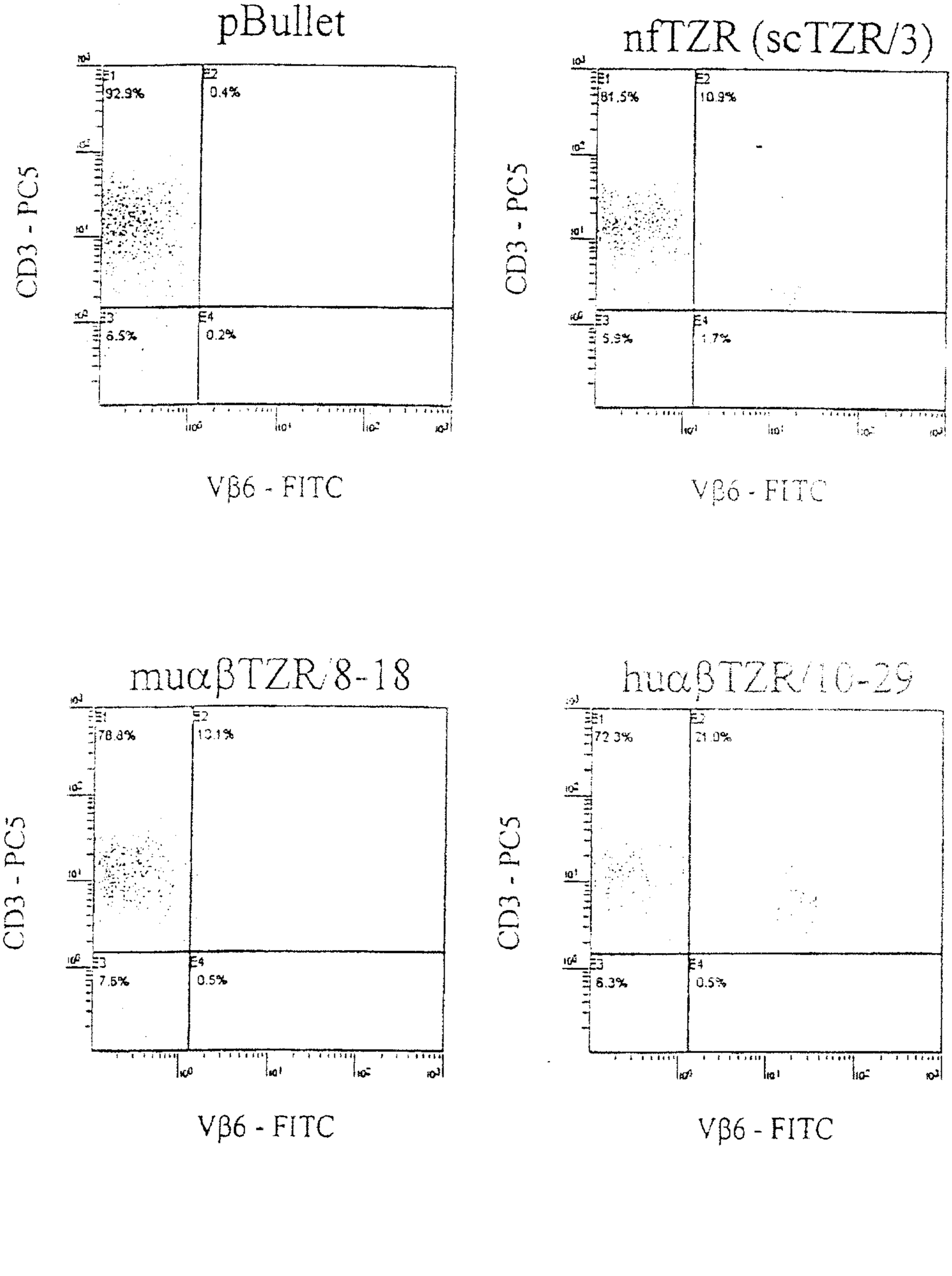
muvahuccαTCR												huCD3ζ											
Pro	Glu	Ser	Ser	Cys^{226}	Pro	Ser	Ser	Tyr	Leu	Leu	Asp	Gly	Ile	Leu	Gly	Leu	Tyr	Asn	Glu	Leu	Asn	Leu	Gly
CCA	GAA	AGT	TCC	TGT	CCC	AGC	TCG	TAC	CTG	GAC	GAT	GGA	ATC	CTC	GGA	CTT	TAC	AAC	GAA	CTC	AAC	CTC	GGA
GGT	CTT	TCA	AGG	ACA	GGG	TCG	AGC	ATG	GAC	GAC	CTA	CCT	TAG	GAG	CCT	GAA	ATG	TTG	CTT	GAG	TTG	GAG	CCT

Figure 8

Fusion of muvβhucβTCR (hdm2 81-88) with huCD3ζ

muvβhucβTCR							huCD3ζ						
Ala	Trp	Gly	Arg	Ala	Asp	$\underline{Cys}^{261}$	Tyr	Leu	Leu	Asp	Gly	Ile	Leu
GCC	TGG	GGT	AGA	GCA	GAC	TGT	TAC	CTG	CTG	GAT	GGA	ATC	CTC
CGG	ACC	CCA	TCT	CGT	CTG	ACA	ATG	GAC	GAC	CTA	CCT	TAG	GAG

Figure 9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Vβ6 - FITC
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92.9%
R2
0.4%
R3
6.5%
R4
0.2%
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CD3 - PC5
Vβ6 - FITC
R1
81.5%
R2
10.9%
R3
5.9%
R4
1.7%
muαβTZR/8-18
CD3 - PC5
Vβ6 - FITC
R1
78.8%
R2
13.1%
R3
7.5%
R4
0.5%
huαβTZR/10-29
CD3 - PC5
Vβ6 - FITC
R1
72.3%
R2
21.0%
R3
6.3%
R4
0.5%

Figure 10

Peptide titration nTTZR (scTTZR/3) - transduced PBLs after Vβ6 selection
in the ^{51}chromium release test

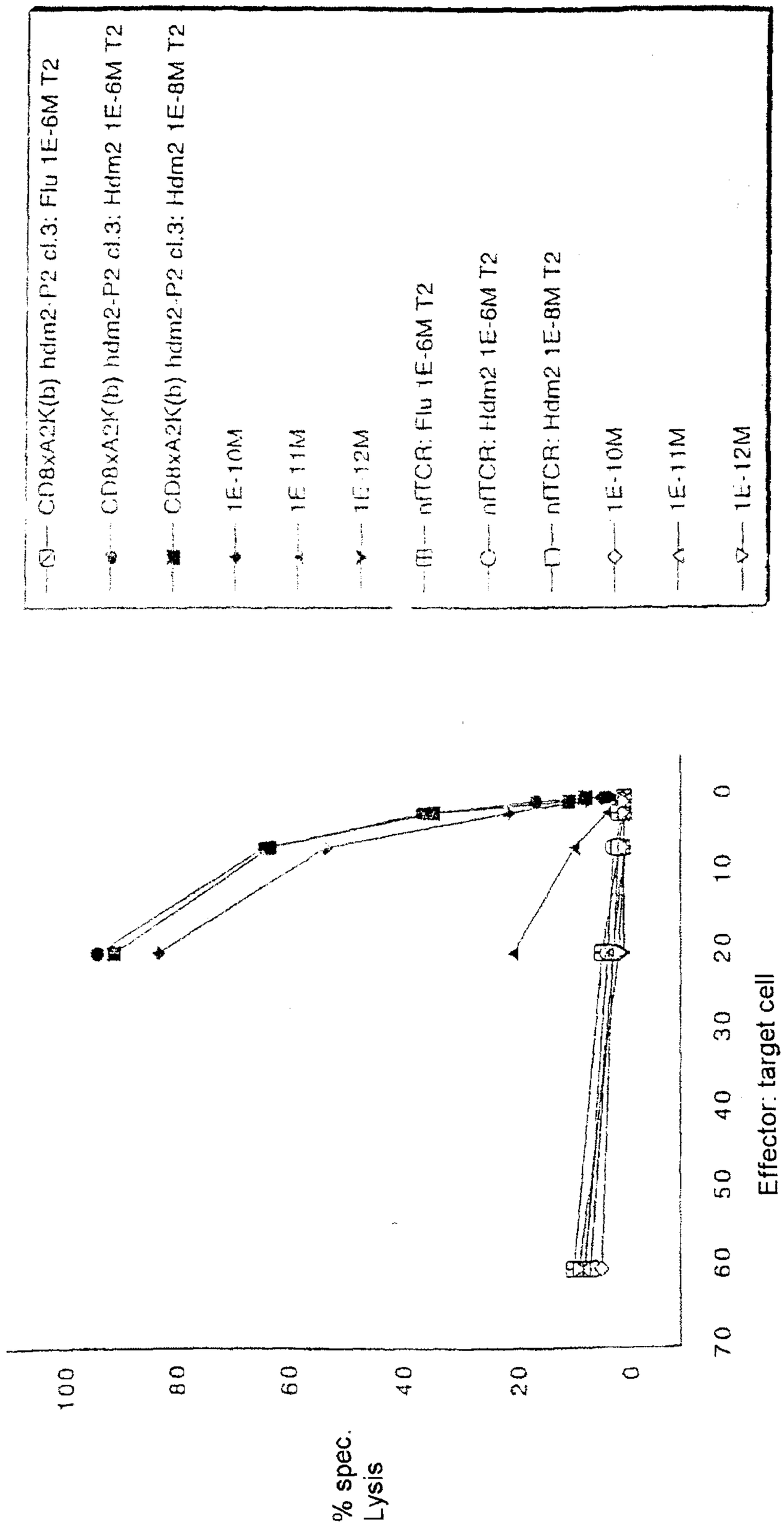


Figure 11

Peptide titration muαβTZR/8-18 - transduced PBLs after vβ6 selection
in the ^{51}chromium release test

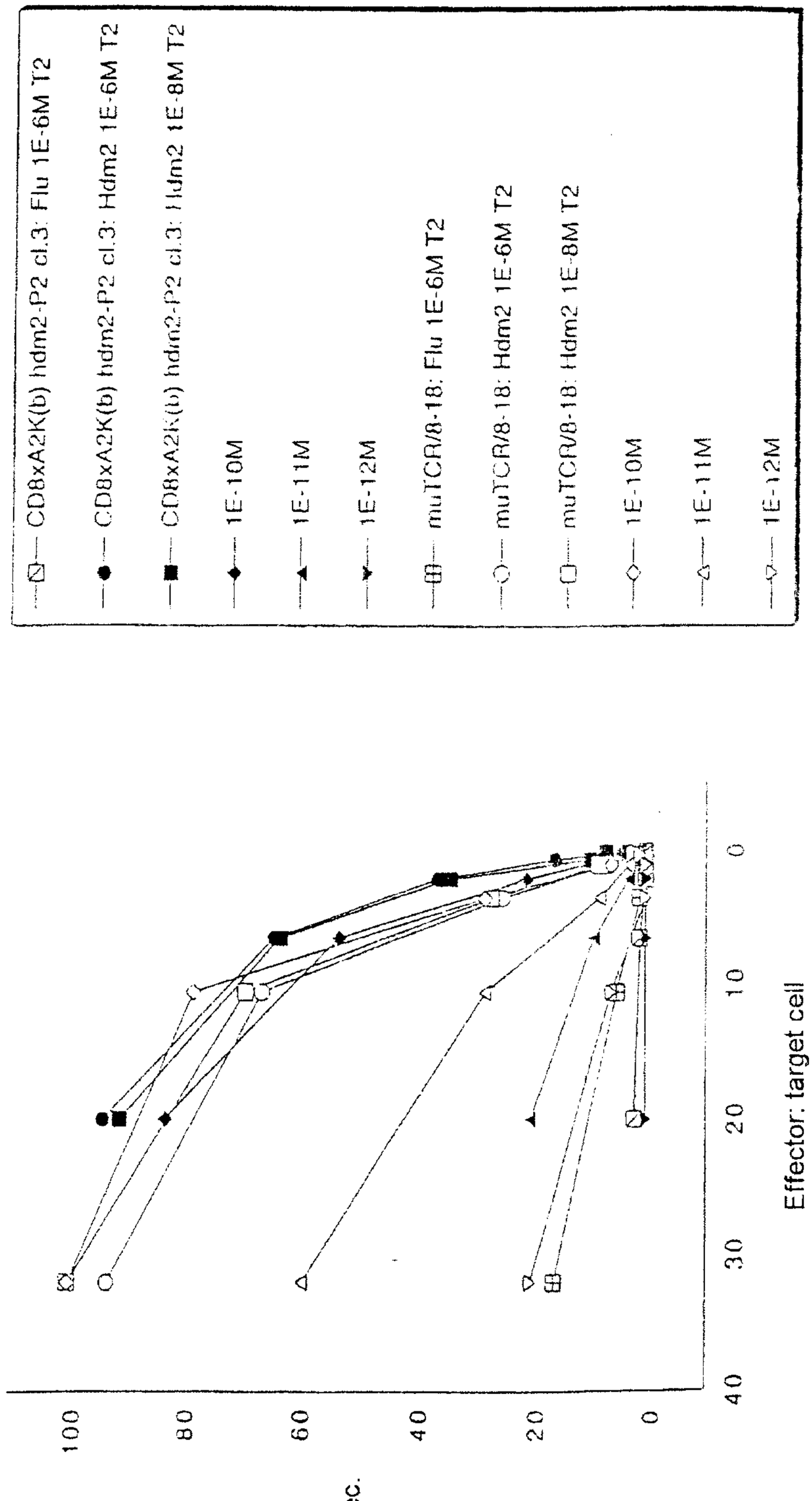


Figure 12

Peptide titration huαβTZR/10-29 transduced PBLs after Vβ6 selection in the ^{51}Cr release test

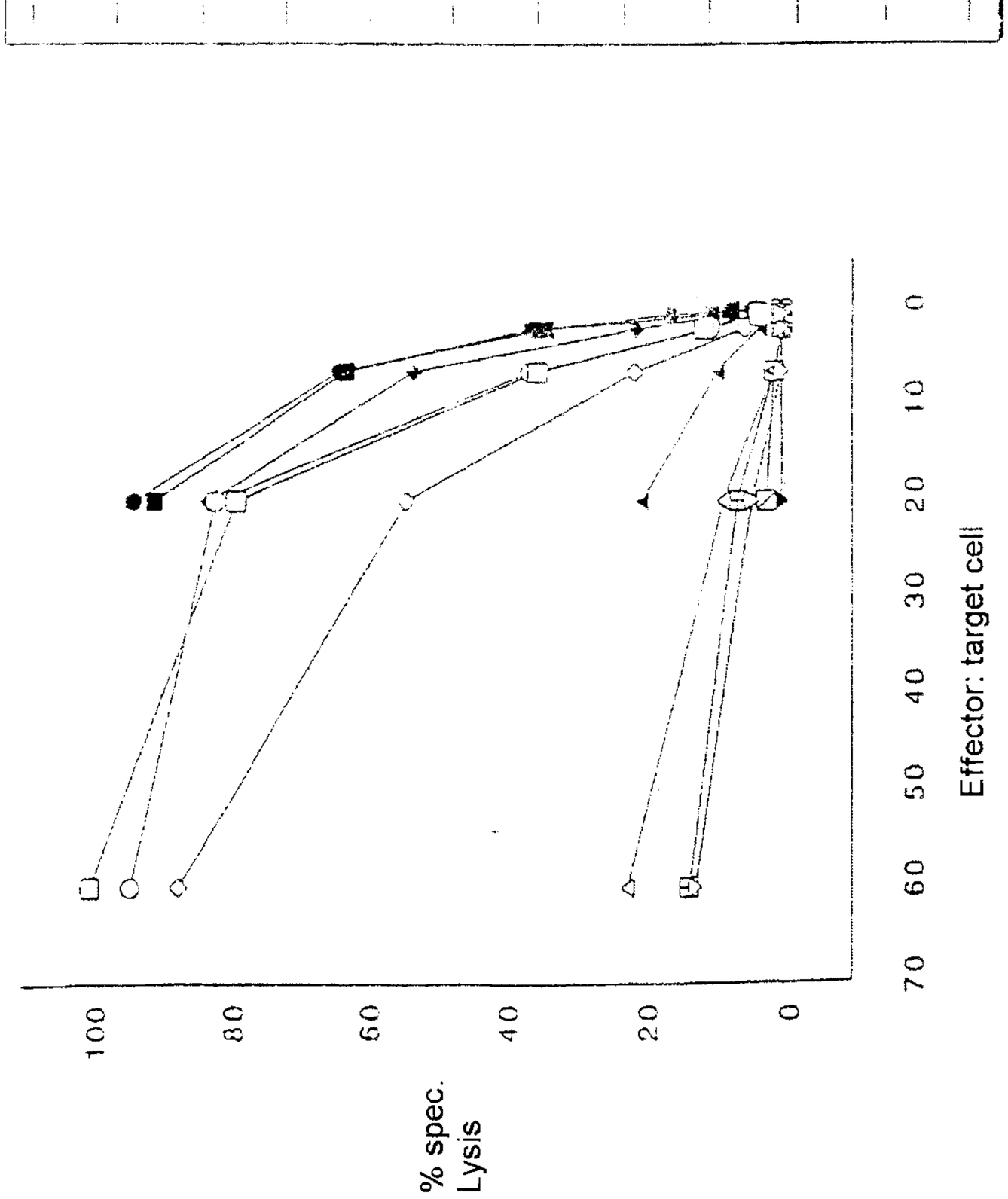


Figure 13

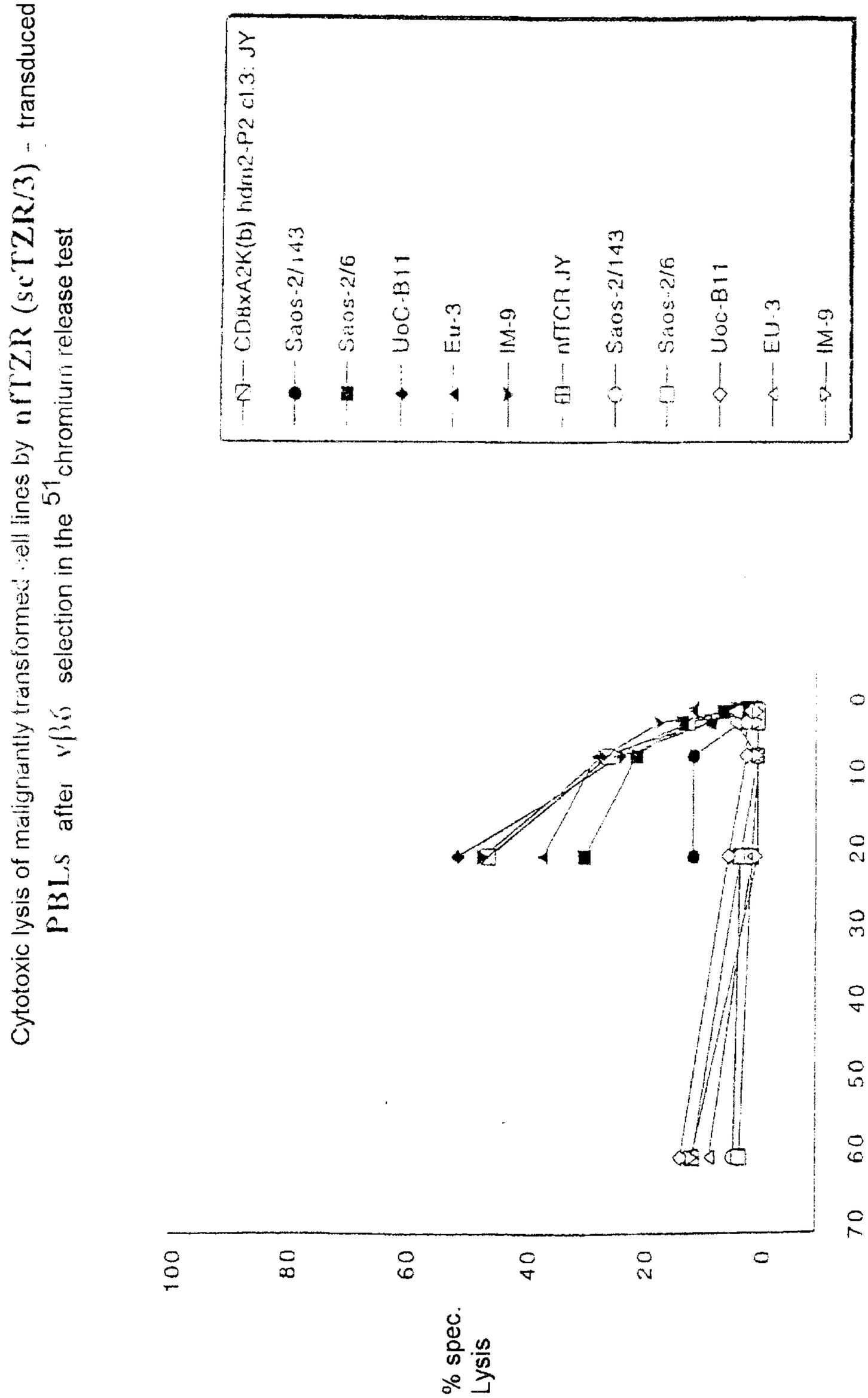

Cytotoxic lysis of malignantly transformed cell lines by nfTZR (scTZR/3) - transduced PBLs after vβ6 stimulation in the 51chromium release test
CD8xA2K(b) hdm2-P2 cl.3: JY
Saos-2/143
Saos-2/6
UoC-B11
Eu-3
IM-9
nfTCR JY
Saos-2/143
Saos-2/6
Uoc-B11
EU-3
IM-9
% spec. Lysis
100
80
60
40
20
0
70
60
50
40
30
20
10
0
Effector: target cell

Figure 14

Cytotoxic lysis of malignant T cell lines by $mu\alpha\beta TZR/8\text{-}18$ - transduced PBLs after $\nu\beta6$ selection in the ^{51}chromium release test

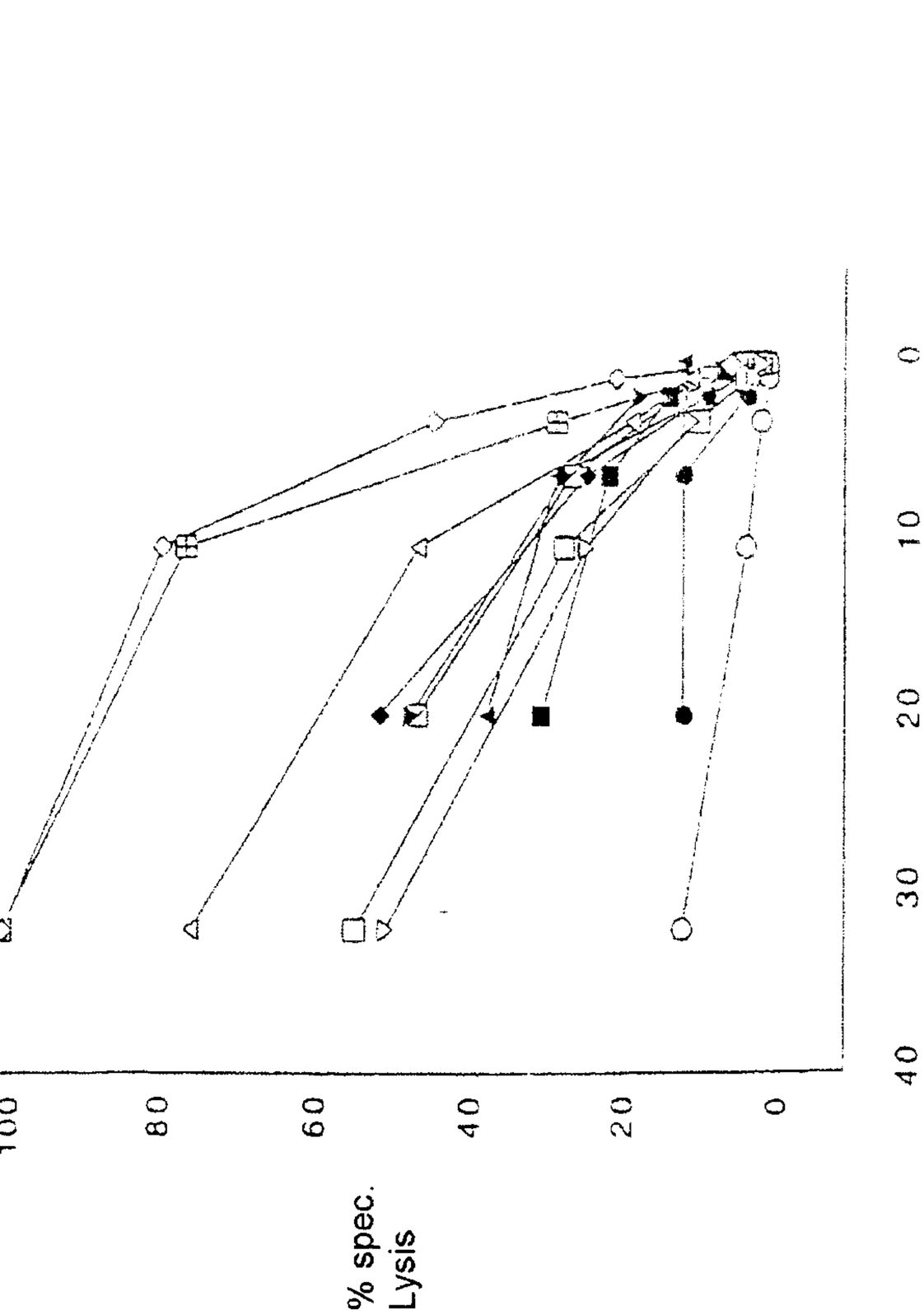
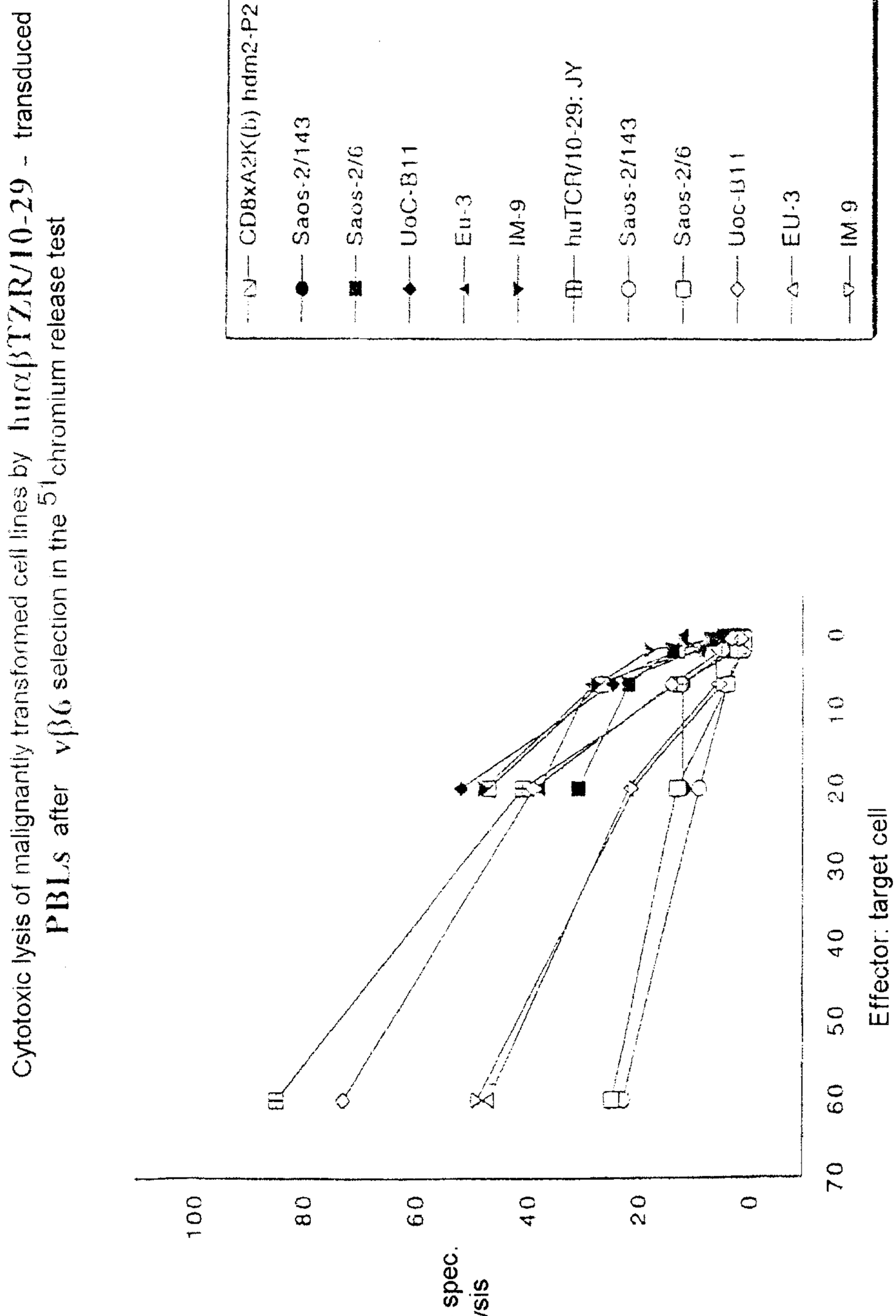
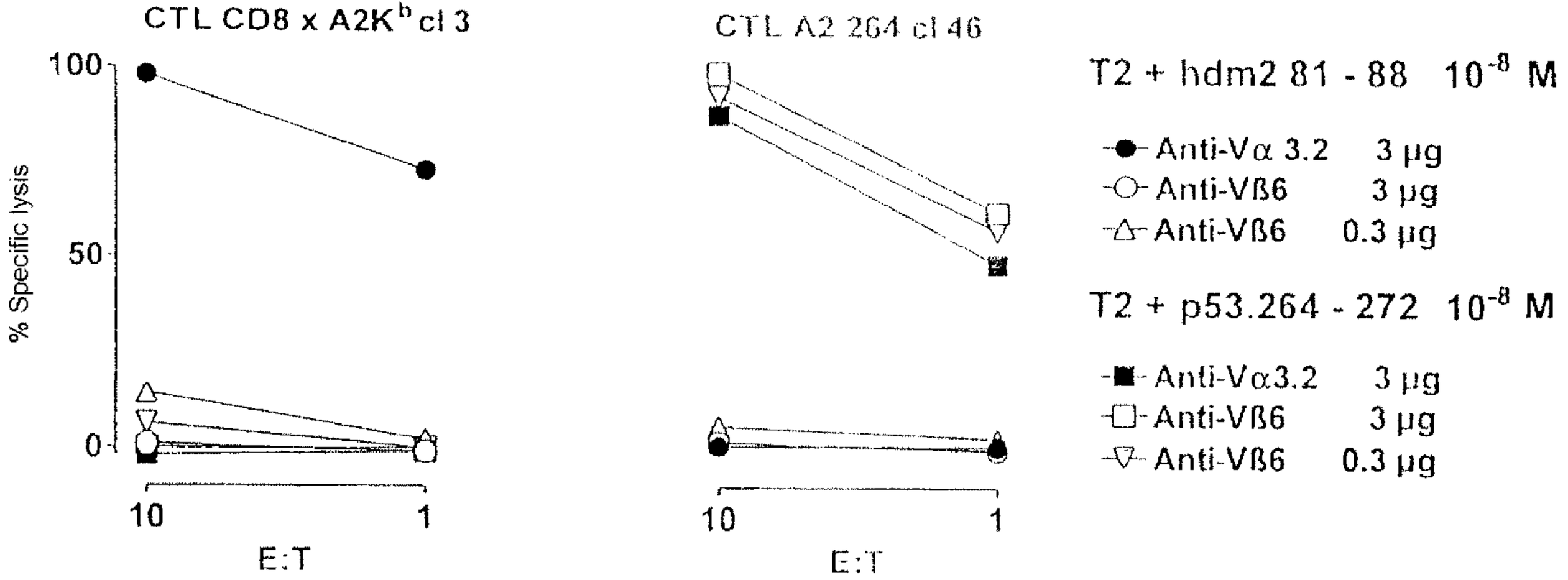


Figure 15


Cytotoxic lysis of malignantly transformed cell lines by huαβTZR/10-29 - transduced PBLs after vβ6 selection in the 51chromium release test
% specific Lysis
100
80
60
40
20
0
70
60
50
40
30
20
10
0
Effector: target cell
CD8xA2K(b) hdm2-P2 cl.3: JY
Saos-2/143
Saos-2/6
UoC-B11
Eu-3
IM-9
huTCR/10-29: JY
Saos-2/143
Saos-2/6
Uoc-B11
EU-3
IM-9


CTL CD8 x A2K^b cl 3
100
50
0
% Specific lysis
10
1
E:T
CTL A2 264 cl 46
10
1
E:T
T2 + hdm2 81 - 88 10^{-8} M
Anti-Vα 3.2 3 µg
Anti-Vß6 3 µg
Anti-Vß6 0.3 µg
T2 + p53.264 - 272 10^{-8} M
Anti-Vα3.2 3 µg
Anti-Vß6 3 µg
Anti-Vß6 0.3 µg