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(57) Abstract

Immune cells having predefined specificity are obtained by either complexing the cells with an antigen–specific MHC–restricted chimeric T cell receptor (TCR) or a fragment thereof, or transfecting said cells with an antigen–specific MHC–restricted chimeric TCR gene. The chimeric TCR comprises: (i) a first segment comprising either (a) a single–chain TCR (scFv–TCR) made of the variable (V) region and, optionally, of either the extracellular constant (C) region of an antigen–specific TCR, or of the constant region of the immunoglobulin kappa light chain (Ck); or (b) a two–chain TCR (tcFv–TCR) made of the extracellular variable (V) and constant (C) regions of an antigen–specific TCR; and (ii) a second segment comprising a signal transducing element of an immune cell. The immune cells can be used for example for the treatment of cancer, infectious diseases, autoimmune diseases or graft rejection.

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IMMUNE CELLS HAVING PREDEFINED BIOLOGICAL SPECIFICITY, COMPRISING CHIMERIC T CELL RECEPTOR

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates generally to the fields of immunology and molecular cell biology. More specifically, the present invention relates to immune cells complexed with a chimeric T cell receptor (TCR) or a fragment thereof, or transfected with a chimeric TCR gene.

Description of the Related Art

The immune response is a complex defense system able to recognize and kill invading organisms, e.g., bacteria, viruses, fungi, allogeneic tissue and some types of tumor cells. The characteristic aspects of the immune system are the specific recognition of antigens, the ability to discriminate between self and non-self antigens and a "memory" which enables a quick, specific reaction to previously encountered antigens. The immune system in mammals reacts to antigens via both humoral and cell-mediated immune responses.

The major pathway of the immune defense begins with the trapping of the antigen by accessory cells such as dendritic cells or macrophages with subsequent concentration in lymphoid organs. Upon specific recognition of the processed antigen, mature T helper cells can be triggered to become activated T helper cells. These activated T helper cells regulate both the humoral immune response by inducing the differentiation of B cells to antibody-producing plasma cells and control of cell-mediated immune response by activation of cytotoxic T lymphocytic (CTL) cells and natural killer (NK) cells.

T lymphocytes recognize antigen in the context of self Major Histocompatibility Complex (MHC) molecules by means of the T cell receptor (TCR). The TCR expressed on the surface of T cells is a disulfide-linked heterodimer non-covalently associated with an invariant structure, the CD3 complex. CD3 is assumed to be responsible for intracellular signalling following occupancy of the TCR by ligand. Most T cells carry TCRs consisting of α and β glycoproteins (α and β chains of the TCR), while some T cells carry TCRs having γ and δ glycoproteins (γ and δ chains).

Like the immunoglobulin (Ig) genes, the TCR genes are composed of variable

segments which rearrange during T cell development. TCR, like Ig polypeptides, consist of amino terminal variable (V) and carboxyl terminal constant (C) regions. The variable region V is responsible for the specific recognition of antigen whereas the constant C region functions in membrane anchoring and in transmitting of the signal that the receptor is occupied, from the outside to the inside of the cell. The variable regions of the Ig heavy chain and the TCR β chain are encoded by three gene segments, the variable (V), diversity (D) and joining (J) segments. The Ig light chain and the TCR α chain contain variable regions encoded by V and J segments.

The T cell receptor for antigen-CD3 complex (TCR/CD3) recognizes antigenic peptides that are presented to it by the MHC proteins. Complexes of MHC and peptide are expressed on the surface of antigen presenting cells and other T cell targets. Stimulation of the TCR/CD3 complex results in activation of the T cell and a consequent antigen-specific immune response. The TCR/CD3 complex plays a central role in the effector function and regulation of the immune system.

Two forms of T cell receptor for antigen are expressed on the surface of T cells. These contain either α/β heterodimers or γ/δ heterodimers. Accordingly, each of these chains is made of the V and C regions of the TCR, namely $V_\alpha C_\alpha$, $V_\beta C_\beta$, $V_\gamma C_\gamma$, and $V_\delta C_\delta$ .T cells are capable of rearranging the genes that encode the α, β, γ and δ chains of the T cell receptor. T cell receptor gene rearrangements are analogous to those that produce functional immunoglobulins in B cells and the presence of multiple variable and joining regions in the genome allows the generation of T cell receptors with a diverse range of binding specificities. Each α/β or γ/δ heterodimer is expressed on the surface of the T cell in association with four invariant peptides. These are the γ, δ and ε subunits of the CD3 complex and the zeta chain. The CD3 γ, δ and ε polypeptides are encoded by three members of the immunoglobulin supergene family and are found in a cluster on human chromosome 11 or murine chromosome 9. The zeta chain gene is found separately from other TCR and CD3 genes on chromosome 1 in both the mouse and human. The CD3 chains and the zeta subunit do not show variability, and are not involved directly in antigen recognition.

All the components of the T cell receptor are membrane proteins and consist of a leader sequence, externally-disposed N-terminal extracellular domains, a single membrane-spanning domain, and cytoplasmic tails. The α, β, γ and δ antigen-binding

polypeptides are glycoproteins. The zeta chain hasa relatively short ectodomain of only nine amino acids and a long cytoplasmic tail of approximately 110 amino acids. Most T cell receptor α/β heterodimers are covalently linked through disulphide bonds, but many γ/δ receptors associate with one another non-covalently. The zeta chain quantitatively forms either disulphide-linked ζ-η heterodimers or zeta-zeta homodimers.

Another example of a type of receptor on cells of the immune system is the Fc receptor. The interaction of antibody-antigen complexes with cells of the immune system results in a wide array of responses, ranging from effector functions such as antibody-dependent cytotoxicity, mast cell degranulation, and phagocytosis to immunomodulatory signals such as regulating lymphocyte proliferation, phagocytosis and target cell lysis. All these interactions are initiated through the binding of the Fc domain of antibodies or immune complexes to specialized cell surface receptors on hematopoietic cells. It is now well established that the diversity of cellular responses triggered by antibodies and immune complexes results from the structural heterogeneity of Fc receptors (FcRs).

FcRs are defined by their specificity for immunoglobulin isotypes. Fc receptors for IgG are referred to as $Fc_\gamma R$, for IgE as $Fc_\varepsilon R$, for IgA as $Fc_\alpha R$, etc. Structurally distinct receptors are distinguished by a Roman numeral, based on historical precedent. We now recognize three groups of $Fc_\gamma Rs$, designated $Fc_\gamma RI$, $Fc_\gamma RII$ and $Fc_\gamma RIII$. Two groups of $Fc_\varepsilon R$ have been defined; these are referred to as $Fc_\varepsilon RI$ and $Fc_\varepsilon RII$. Structurally related although distinct genes within a group are denoted by A, B, C. Finally, the protein subunit is given a Greek letter, such as $Fc_\gamma RIIIA_\alpha$, $Fc_\gamma RIIIA_\gamma$. All the CD3 chains (γ, δ, ε, ζ and η) and the FcR-γ chain contain Immunoreceptor Tyrosine Activation Motifs (ITAMs) which undergo phosphorilation and is responsible for the signal transduction of the cells.

Considerable progress has been made in defining the heterogeneity for IgG and IgE Fc receptors ($Fc_\gamma R$, $Fc_\varepsilon R$) through their molecular cloning. Those studies make it apparent that Fc receptors share structurally related ligand binding domains, but differ in their transmembrane and intracellular domains which presumably mediate intracellular signalling. Thus, specific $Fc_\gamma Rs$ on different cells mediate different cellular responses upon interaction with an immune complex. The structural analysis of the $Fc_\gamma Rs$ and $Fc_\varepsilon RI$ has also revealed at least one common subunit among some of these receptors. This common subunit is the γ subunit, which is similar to the ζ or η chain of the

TCR/CD3, and is involved in the signal transduction of the $Fc_{\gamma}RIII$ and $Fc_{\varepsilon}RI$ through its ITAM motif.

The low affinity receptor for IgG ($Fc_{\gamma}RIIIA$), is composed of the ligand binding $CD16_{\alpha}$ ($Fc_{\gamma}RIIIA_{\alpha}$) polypeptide associated with the γ chain ($Fc_{\gamma}RIIIA_{\gamma}$). The CD16 polypeptide appears as membrane anchored form in polymorphonuclear cells and as transmembrane form ($CD16_{TM}$) in NK cells. The $Fc_{\gamma}RIIIA$ serves as a triggering molecule for NK cells.

Another type of immune cell receptor is the IL-2 receptor. This receptor is composed of three chains, the α chain (p55), the β chain (p75) and the γ chain. When stimulated by IL-2, lymphocytes undergo proliferation and activation.

Antigen-specific effector lymphocytes, such as tumor specific T cells (Tc), are very rare, individual-specific, limited in their recognition spectrum and difficult to obtain against most malignancies. Antibodies, on the other hand, are readily obtainable, more easily derived, have wider spectrum and are not individual-specific. The major problem of applying specific antibodies for cancer immunotherapy lies in the inability of sufficient amounts of monoclonal antibodies (mAb) to reach large areas within solid tumors. In practice, many clinical attempts to recruit the humoral or cellular arms of the immune system for passive anti-tumor immunotherapy have not fulfilled expectations. While it has been possible to obtain anti-tumor antibodies, their therapeutic use has been limited so far to blood-borne tumors primarily because solid tumors are inaccessible to sufficient amounts of antibodies. The use of effector lymphocytes in adoptive immunotherapy, although effective in selected solid tumors, suffers on the other hand, from a lack of specificity (such as in the case of lymphokine-activated killer cells (LAK cells) which are mainly NK cells) or from the difficulty in recruiting tumor-infiltrating lymphocytes (TILs) and expanding such specific T cells for most malignancies. Yet, the observations that TILs can be obtained in melanoma and renal cell carcinoma tumors, that they can be effective in selected patients and that foreign genes can function in these cells demonstrate the therapeutic potential embodied in these cells.

PCT WO/94/29438 describes a method for transducing mammalian target cells with foreign genes, e.g. a gene encoding a chimeric T cell receptor, comprising transient cotransfection of a first population of mammalian cells that can produce virus with a retroviral packaging plasmid and a retroviral vector encoding a foreign gene, and cocultivation of said first population of mammalian cells producing replication-defective

recombinant retroviral vectors carrying said foreign gene with a second population of mammalian target cells to transduce said second population of target cells with said foreign gene. The TCR contains a non-MHC restricted extracellular surface membrane protein domain binding specifically to at least one ligand.

The prior art is deficient in the lack of effective means of treating cancer and effectively treating a wide variety of immunologically based disease conditions, particularly autoimmune diseases and graft rejection.

SUMMARY OF THE INVENTION

It is an object of the present invention is to endow immune cells with a predefined specificity by grafting immune cells with chimeric TCR specificity.

The present invention relates to an immune cell having predefined specificity, wherein said immune cell is either complexed with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) or a fragment thereof, or is transfected with an antigen-specific MHC-restricted chimeric TCR gene.

In one embodiment, the immune cell is complexed with the TCR or TCR fragment by chemical conjugation. The TCR or TCR fragment may also be bridged to the immune cell by a macromolecule linker such as, but not being limited to, avidin, streptavidin or polylysine, or by a bispecific antibody that binds both to the TCR or TCR fragment and to the immune cell.

In another embodiment, the immune cell is transfected with a chimeric TCR gene or a DNA sequence encoding a chimeric TCR, said chimeric TCR comprising: (i) a first segment encoding either a single-chain TCR (scFv-TCR) made of the variable (V) region and, optionally, of the extracellular constant (C) region, of the TCR, or a two-chain TCR (tcFv-TCR) made of the extracellular variable (V) and constant (C) regions of the TCR, and (ii) a second segment encoding a signal transducing element of an immune cell. The scFv-TCR may be the alpha and the beta, or the gamma and the delta, chains of the antigen-specific TCR.

In one embodiment of the invention, the said first segment encodes a two-domain (2D) single chain TCR made of the extracellular variable (V) region $V\alpha V\beta$chains of the TCR linked by a linker. In another embodiment, the said first segment encodes a three-domain (3D) single chain TCR made of the extracellular variable (V) and constant (C) regions of the TCR. Such as a single-chain TCR comprising the $V\alpha V\beta C\beta$ or $V\alpha V\beta C\alpha$ chains of said

antigen-specific TCR. In a further embodiment, the first segment is a three-domain (3D) single-chain TCR made of the extracellular variable (V) $V\alpha V\beta$ chains of said antigen-specific TCR and the constant (C) region of the immunoglobulin kappa light chain (Ck).

In a further embodiment, the said first segment encodes a two-chain TCR (tcFv-TCR) made of the extracellular variable (V) and constant (C) regions $V\alpha$ $C\alpha$+ $V\beta C\beta$ chains of the TCR.

The linker that links the $V_\alpha V_\beta$ chains is selected from standard linkers known in the art such as oligopeptides of 15-20 amino acids that allow flexibility and proper association between the two V domains.

The signal transducing element may be comprised of the intracellular signalling unit only or of the intracellular signalling unit plus transmembrane domain or of these both and extracellular domain of signalling chains. Examples used according to the invention include, but are not limited to, the whole molecules or part of the molecules selcted from TCR/CD3 complex gamma, delta, epsilon, eta or zeta chain, the gamma chain of the Fc receptor, the alpha, beta and/or gamma subunit of the IL-2 receptor, the CD2, CD16 and CD28 co-receptor chains, the signaling elements of the natural killer (NK) receptor, killing inhibitory receptor (KIR) or killing activating receptor (KAR), or any signaling element derived from an immune cell, and combinations of said signal transducing elements.

Repertoires of T cell receptor fragment genes can be derived from natural sources such as peripheral blood lymphocytes (PBLs), tumor infiltrating T-cells or cloned cytotoxic T cells or cell lines, or can be derived from TCR V-gene segments created in part or completely synthetically. The scFv-TCR or tcFv-TCR can be cloned from immune T lymphocytes or from synthetic libraries such as, but not limited to, phage display libraries, viral display libraries or others. The variable regions of TCRs can be obtained from human and non-human species T lymphocytes.

Another object of the present invention is to provide techniques enabling the construction of chimeric TCR chimerized to a signaling element and the introduction of these scFv/ or tcFv/signal transducing molecule into mammalian cells to provide a desired biological action in such cells at a target cell or organ site. The transfection of the immune cells with the TCR genes may be performed by any standard method including, but not being limited to, with viral vectors, eukaryotic vectors, and electrical or

chemical means such as calcium phosphate transfection, dextrane sulphate transfection, liposomal transfection and electroporation.

Still another object of the present invention is to provide techniques enabling the construction of proteins which recognize MHC-presented antigens chimerized to a signaling element.

The immune cell is preferably human. Various types of lymphocytes and non-lymphocytic cells may be suitable, for example, resting, activating and memory T lymphocytes, cytotoxic T lymphocytes (CTLs), helper T cells, natural killer (NK) cells, tumor-infiltrating lymphocytes (TIL), non-T lymphocytes, B cells, plasma cells, monocytes, macrophages, eosinophils and dendritic cells, subtypes thereof and any other cell type which can express chimeric receptor chain. Both antigen-specific and non-specific immune cells are contemplated by the invention.

In one embodiment, the immune cell is an antigen-processing cell and said predefined specificity is targeted antigen uptake and presentation.

In another embodiment, the immune cell is selected from the group consisting of lymphocytes, NK cells, macrophages, monocytes, eosinophils and other cytotoxic cells and said predefined specificity is target cell cytotoxicity.

In a further embodiment, the immune cell is a helper T cell and said predefined biological specificity is increased lymphokine or cytokine production.

In still another embodiment, the immune cells are B lymphocytes or plasma cells and said predefined specificity is increased immunoglobulin production.

In yet a further embodiment, the immune cells are T cells, B cells, monocytes and macrophages and said predefined biological specificity is increased antigen-presenting functions.

In another further embodiment, the immune cell is a target cell immune cell specific for target cell, said immune cell containing an antigen-binding molecule selected from the group consisting of the alpha/beta chains or the gamma/delta chains of the antigen-specific T cell receptor, chimerized to a signal transducing element selected from the group comprising the TCR/CD3 complex gamma, delta, epsilon, eta or zeta chain, the gamma chain of the Fc receptor, the alpha, beta and/or gamma subunit of the IL-2 receptor, the CD2, CD16 and CD28 co-stimulatory chains, the signaling elements of the natural killer (NK) receptor, killing inhibitory receptor (KIR) or killing activating receptor (KAR), or any signaling element derived from an immune cell, and combinations of said signal

transducing elements.

The antigen-binding molecule may bind to an antigen including, but not being limited to, viral antigens, synthetic antigens, tumor associated antigens, tumor specific antigens, mucosal antigens, superantigens, differentiation antigens, auto-immune antigens, class I MHC molecules and class II MHC molecules. In one embodiment, the antigen-binding molecule binds to the melanoma-associated neoplastic protein (MAGE-1) antigen.

According to the invention, target cell antigen-specific immune and non-immune cells can be created to identify on mammalian cells the MHC-restricted antigenic structure(s) recognized by these relevant chimeric scFv-TCR or tcFv-TCR. In addition, a technique is provided to identify whether particular antigens against which said TCR are directed are expressed by mammalian cells *in vitro* or *in vivo*.

The present invention further relates to pharmaceutical compositions comprising the immune cells of the invention, which may comprise a pharmaceutically acceptable carrier. These pharmaceutical composition may be used for the treatment of cancer, infectious diseases, autoimmune diseases or graft rejection. The pharmaceutical composition comprising immune cells containing an antigen-binding molecule that binds MAGE-1, are suitable for the treatment of melanoma.

The invention further provides a method for treatment of a tumor in a patient comprising transfecting lymphocyte cells of the patient with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) gene containing a segment encoding a scFv-TCR or tcFv-TCR which binds to an antigen associated with said tumor and a segment encoding a signal transducing element of an immune cell.

Still further provided is a method of treatment of melanoma in a patient comprising transfecting lymphocyte cells of the patient with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) gene containing a segment encoding a scFv-TCR or tcFv-TCR which binds to the melanoma-associated neoplastic protein (MAGE-1) antigen and a segment encoding a signal transducing element of an immune cell.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A schematically represents a chimeric single chain TCR $V_\alpha/V\beta\text{-}\gamma$ [(scFv)-TCR/$_\gamma$] at protein level; **Fig. 1B** schematically represents some configurations of chimeric TCR constructs: (*i*) chimeric single chain (ch-sc) TCR $V_\alpha V\beta C\beta\zeta$ genes; (*ii*)

chimeric two chain (ch-tc) TCR $\alpha\zeta$ genes; *(iii)* ch-tc TCR $\beta\zeta$ genes; (*iv*) full-length TCR α-chain genes; and *(v)* full-length TCR β-chain genes (L, leader; V, variable region; J, joining region; D, diversity region; C, constant region; EC, extracellular region; TM, transmembrane region; Cy, Cytoplasmic region; Li, linker).

Fig. 2 shows the sequences of the V_α (2A) and V_β (2B) chains of the TCR from the MAGE-1 specific CTL clone 82/30, and of the primers used for amplification of the V_α and V_β chains.

Fig. 3 shows restriction sites used in the construction of the single chain TCR L domain.

Figs. 4A-4B show the construction of the retroviral vector constructs.

Figs. 5A-5H illustrate expression of (chimeric-) TCR constructs on primary human T lymphocytes. Expression of (*i*) chimeric single chain TCR $V_\alpha V_\beta C_\beta\zeta$, (*ii*) chimeric two chain $V_\alpha C_\alpha\zeta$ /$V_\beta C_\beta\zeta$, and (*iii*) full-length TCR $\alpha\beta$ constructs was determined by flow cytometry after gene transduction of primary human T lymphocytes. Events shown represent viable lymphocytes stained with anti-$V_\alpha 12.1^{FITC}$ and anti-$V_\beta 1^{PE}$. **(5A):** Human T lymphocytes transduced with the CD24 gene (negative control). **(5B):** HLA-A1 restricted, MAGE-1 specific parental CTL clone MZ2-82/30 (positive control). **(5C):** Human T lymphocytes transduced with $V_\alpha V_\beta C_\beta$-ζ retroviral vectors. **(5D):** Human T lymphocytes transduced with $V_\alpha V_\beta C_\beta$-ζ retroviral vectors and enriched by flow sorting using anti-$V_\alpha 12.1$ FITC and anti-V_β-1 PE mAbs. **(5E):** Human T lymphocytes transduced with $V_\alpha C_\alpha$-ζ and $V_\beta C_\beta$-ζ retroviral vectors simultaneously. **(5F):** Human T lymphocytes transduced with $V_\alpha C_\alpha$-ζ and $V_\beta C_\beta$-ζ retroviral vectors and followed by enrichment by flow sorting using anti-$V_\alpha 12.1$ FITC and anti-V_β-1 PE mAb. **(5G):** Human T lymphocytes transduced with full-length TCR α and β retroviral vectors simultaneously. **(5H):** Human T lymphocytes transduced with full-length TCR α and β retroviral vectors, followed by $V_\alpha 12.1$ immunomagnetic selection and a second enrichment by flow sorting using soluble multimeric HLA-A1/MAGE-1/$Strep^{PE}$ complexes.

Fig. 6 shows that chimeric (scFv)-TCR_γ or ζ^{POS} T lymphocytes are able to specifically bind to soluble multimeric HLA-a1/MAGE-1 complexes. Complexes with an irrelevant peptide derived from influenza virus nucleoprotein were not bound by the chimeric (scFv)-TCR_γ or ζ^{POS} T lymphocytes.

Fig. 7 shows that peptide pulsed target cells are lysed by (chimeric-) TCR

transduced human T lymphocytes. MAGE-1 peptide-pulsed HLA-A1^{pos} target cells were incubated with the indicated human T lymphocytes and tested in a 6-h ^{51}Cr release assay. Left row and middle row show the data using ^{51}Cr labeled HLA- A1^{pos}, MAGE-1^{neg} EBV transformed B cell lines 72-2 and APD respectively, pulsed with MAGE-1 or Influenza virus peptides (10 μg/ml). The right row shows the data using ^{51}Cr labeled HLA-A1^{pos}, MAGE-1^{neg} MEL 2A melanoma cells pulsed with MAGE-1 or influenza virus peptides (10 μg/ml). Experiments were performed in triplicate, and the SD did not exceed 10%. The per cent specific ^{51}Cr release is shown at different E:T ratios.

Figs. 8A-8E shows that native HLA-A1^{POS}, MAGE-1^{POS} melanoma cells are susceptible to lysis by (chimeric-) TCR transduced T lymphocytes. Cytotolytic capacity of human T lymphocytes was determined in 6-h ^{51}Cr release assays. Left two rows show results obtained with ^{51}Cr labeled HLA-A1^{POS}, MAGE-1^{POS} melanoma cells (MZ2-MEL 3.0 and 518A2). The right row shows results obtained with control ^{51}Cr labeled HLA-A1^{NEG}, MAGE-1^{NEG} melanoma cells (MEL 78 — , BLM - - - -) and renal cell carcinoma cells (SKR17 cl4 -----). Anti-HLA-ABC (10μg/ml, ----------) or mouse Ig (10μg/ml _ _ _) was added to the target cells 15-30 min. prior to incubation with the effector T lymphocytes (——, no mAb). Shown are mean percentages of triplicates of per cent specific ^{51}Cr release. Data of one representative experiment out of 3 are shown.

Fig. 9 shows that soluble multimeric HLA-A1/MAGE-1 complexes identify functional CTL expressing retroviral introduced chimeric 3D scFv-TCR and chimeric tc-TCR and full-length TCR αβ transgenes. Functional characteristics of human T lymphocytes were determined in flow cytometry and ^{51}Cr release assays as described in materials and methods. **(A)**: Flow cytometric analysis of human T lymphocytes incubated with soluble HLA-A1/MAGE-1/Streptavidin^{PE} complexes (A1/M1) and Streptavidin^{PE} (Strep^{PE}) was performed. **(B)**: Cytotoxicity of human T lymphocytes against HLA-A1^{POS}, MAGE-1^{POS} melanoma cells and peptide pulsed HLA-A1^{POS}, MAGE-1^{NEG} EBV transformed B cells was analysed in a 6-h ^{51}Cr release assay. The HLA-A1^{POS}, MAGE-1^{POS} melanoma cell was incubated with the human CTL (- - -). Anti-MHC class I mAb was added (10 μg/ml, - - - - -) to the target and effector cell mixture to demonstrate HLA-A1/MAGE-1 specificity of lysis. The ^{51}Cr labelled HLA-A1^{POS} EBV transformed B cell line APD was pulsed with MAGE-1 peptide (10 μg/ml, ————, no peptide: ·····). **(C)**: Flow cytometric analysis of the human T lymphocytes using anti-V$_\alpha$12.1^{FITC} and anti-V$_\beta$1^{PE} mAbs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to chimeric TCR receptors constructed from the variable regions of the TCR, in particular in the single chain design [(scFv)-TCR], the generation of immune-specific cells such as T lymphocytes, non-T lymphocytes, NK cells, monocytes or macrophages by transfecting these cells with chimeric (single or two gene(s)) T cell receptors.

The targeting of antigen-specific/non-specific immune cells

According to the present invention, chimeric TCRs are constructed by chimerizing the sequenced and cloned scFv-TCR or two-chain-Fv-TCR to any signal transducing molecule selected from immune cells, such as, but not limited to, CD2, CD3, CD16, CD28, γ-chains, ζ-chains, KIR, KAR or fragments thereof, etc., and by molecular engineering and subsequently transfecting human or non-human immune cells (specific or non-specific) with genes encoding for scFv chimeric TCR. The invention is useful to identify whether target cells express the antigen for which the chimeric TCR is specific. The present invention is also useful when it is desirable to use the immune cells expressing the scFv-TCR or tcFv-TCR for immunotherapy of cancer, infectious diseases and autoimmune diseases, including rheumatoid arthritis.

Thus, the present invention provides techniques enabling one with ordinary skill to graft immune cells with chimeric scFv-TCR or tcFv-TCR specificity at will. The purpose of transfecting said immune cells is to make the cells specific towards target cells. That is, the transfected immune cells of the present invention are triggered as a result of binding of the scFv-TCR or tcFv-TCR to provide a specific biological action such as, but not limited to, targeted antigen uptake and presentation (antigen-processing cells), target cell cytotoxicity (T lymphocytes, NK cells, monocytes, etc.), lymphokine or cytokine production (Helper T cells), Ig production (B lymphocytes and plasma cells), and antigen-presenting functions (T cells, B cells, monocytes, macrophages).

The present invention creates target cell antigen-specific immune and non-immune cells to identify on mammalian cells the MHC-restricted antigenic structure(s) recognized by the relevant chimeric scFv-TCR or tcFv-TCR. Generally, a suitable antigen for the purposes outlined here in the present invention is any antigen or target cell membrane expressed molecules against which a T cell response can be mounted *in vitro* or *in vivo* or for which such a scFv-TCR or tcFv-TCR can be selected. Representative examples of

suitable antigens include, but are not limited to, viral antigens, synthetic antigens, tumor-associated antigens (TAA), tumor-specific antigens (TSA), MHC molecules (class I and class II) or autologous antigens. A hallmark of the present invention is that it allows the grafting of the MHC-restricted specificity to immune cells chimerized with a signalling element from immune cells, thereby triggering biological activities in the recipient immune cells following interaction with the relevant target cells. Moreover, chimeric scFv-TCR or tcFv-TCR with specificity for MHC non-restricted antigens such as, but not limited to, mucosal antigens and superantigens, can be deived from natural sources such as PBLs, tumor-infiltrating T cells or cloned cytotoxic T cells or cell lines, or can be derived from TCR V-gene segments created synthetically, chimerized to said signal transducing molecules and transfected into these immune cells.

Autologous, allogeneic or xenogeneic cytotoxic T lymphocytes (CTL) can often be generated against 'non'- or weak lymphocytes, TAA, TSA or viral epitopes, differentiation antigens or other (glyco) proteins. On the other hand, antigens which have mutated can act on natural TCR as antagonists to inhibit the CTL response to the wild-type epitope. This leads to T cell anergy and, hence, lack of a host immune response to either tumor TAA, TSA, or virus, leading to e.g., a tumor cell growth or viral persistence. Existing technology now allows the creation of peptides and their presentation to T lymphocytes, antigen-presenting cells, resulting in subsequent effective T lymphocyte response.

The present invention provides (1) selection of scFv- or tcFv-TCR-DNA from natural sources such as PBLs, tumor-infiltrating T cells or cloned cytotoxic T cells or cell lines, or derived from TCR V-gene and, optionally, extracellular C, segments created synthetically, (2) cloning of these scFv- or tcFv-TCR-DNA, and (3) their chimerization to signalling structure and their transfection into immune cells.

The present invention allows the use of said modified immune cells in adoptive *in vivo* gene and/or immunotherapy, to combat cancer, virus infections, or autoimmune diseases. Regarding organ or tissue graft or auto-reactive cells, chimeric scFv-TCR or tcFv-TCR reactive with TCR epitopes on host T cells which cause autoimmunity or graft rejection can be transfected in the host T cells for elimination of the reactive cells following *in vivo* transfer. In practice, a wide range of cloned TCR genes chimerized to genes encoding for immune cell signalling molecules with the requisite specificities can be generated by a person having ordinary skill in this art, cloned into the genome of viruses or other vectors and, with existing technology, introduced into host T cells for autologous

adoptive gene transfer and/or immunotherapy.

The chimeric TCR may also be complexed to the immune cells by chemical, physical and/or immunological means by a person having ordinary skill in the art. It is possible that one with ordinary skill may generate MHC negative immune cells to serve as recipient cells for scFv or tcFv chimeric TCR transfection so, that they can be used as universal immune cells for adoptive gene and/or immunotherapy.

The present invention relates to the functional expression of a cloned scFv-TCR or tcFv-TCR DNA or gene which has been chimerized to signal transducing elements derived from natural sources such as PBLs, tumor-infiltrating T cells or cloned cytotoxic T cells or cell lines into mammalian immune system cells. The invention is applicable to situations where it is desirable to have immune cells with a predetermined specificity in order to, e.g., investigate whether mammalian cells express the relative ligand for that chimeric TCR or introduce these chimeric TCR-expressing cells into immune cells of mammals that suffer from cancer, infectious disease or autoimmune diseases.

The present invention also provides techniques to identify whether particular antigens against which said TCR are directed, are expressed by mammalian cells *in vitro* or *in vivo*. For these and other purposes the invention provides for MHC-restricted antigen-specific immune cells that (following introduction of a scFv- or tcFv- chimeric TCR-gene into these cells) functionally express these receptors on their surface and endows the cells with a predetermined antigen specificity. The antigen-binding substance can be selected from TCR, fragments of TCR and antigen-binding peptides other than antibodies.

The antigen named MAGE-1 (melanoma-associated neoplastic protein) codes for antigens recognized by autologous T lymphocytes on a melanoma tumor. These genes are not expressed in normal tissues except for testis. They are expressed in many tumors of several histological types. Also differentiation antigens coded by genes such as tyrosinase are recognized on human cancer cells by autologous CTL. The identification and characterization of these and other human tumor or viral infected and other tissue cells open the possibility for the systemic approach to specific immunitherapy with the engineered immune cells provided by this invention.

It is important to realize that tumor specific neo-antigens are easily tolerated by the immune system, equivalent of a tissue specific antigen. Therefore, only if tumor specific neo-antigens are effectively processed and presented *in vivo* or *in vitro*, will it serve as a

dominant target for T lymphocyte immunity. Whether or not a tumor-specific neo-antigen is immunogenic also depends on which tissue type and in what context it is expressed.

When a TAA is identified, characterized and cloned, it can be introduced in different tissue types so that it becomes immunogenic and specific T lymphocyte immunity can be generated against it (natural sources). ScFv- or tcFv-TCR as described in this invention can also be derived from natural sources such as PBLs, tumor infiltrating T-cells or cloned cytotoxic T cells or cell lines or can be derived from TCR V-gene and, optionally, extracellular C-gene, segments created in part or completely synthetically, chimerized to signal transducing elements selected from immune cells and then introduced into immune cells.

Table 1 herein shows examples of TCR and their tumor cellular ligands. TCR with specificities are possible candidates for use within the present invention or any system of the epitope specificity. Tumor antigens, viral antigens, MHC-antigens, differentiation antigens, autoimmune antigens and superantigens can be identified using T lymphocytes. T lymphocytes are the critical mediators of tumor specific rejection. Consequently, a wide variety of potential tumor antigens and other antigens recognizable by T cells are applicable. These chimeric scFv-TCR gene-transduced immune cells can be used for *in vivo* immunotherapy of cancer, infectious diseases or autoimmune diseases as described, or for the identification of relevant ligands on other mammalian cells.

Thus, the present invention provides an immune cell having predefined biological specificity, wherein said immune cell is grafted with a chimeric T cell receptor. Generally, the immune cell of the present invention may be made from any human or non-human immune cell. Representative examples of immune cells include T lymphocytes, non-T lymphocytes, B cells, monocytes, macrophages, dendritic cells, NK cells plasma cells and Helper T cells. The immune cell may be either a specific immune cell and a non-specific immune cell.

In a preferred embodiment of the present invention of the mutated immune, the chimeric T cell receptor is a chimera between a single chain Fv-TCR or a two-chain Fv-TCR and a signal transducing element of an immune cell. Representative examples of signal transducing element include CD2, CD3, CD16, CD28, γ-chains, ε-chains, ζ-chains, KIR, KAR and fragments thereof.

There are various preferred embodiments of the present invention. For example, the immune cell could be of an antigen processing and presenting cell, immune cell and the

predefined biological specificity is targeted antigen uptake and presentation. Another embodiment of the present invention provides an immune cell wherein the immune cell is T lymphocyte, B cell macrophage or a monocyte and the predefined biological specificity is target cell cytotoxicity. The immune cell can be a helper T cell or other immune cell and the predefined biological specificity is increased lymphokine or cytokine production.

An alternate form of the immune cell of the present invention is a B lymphocyte or a plasma cell and the predefined biological specificity is increased immunoglobulin production. Another alternate form of the immune cell is a mutated T cell, B cell, monocyte or macrophage and said predefined biological specificity is increased antigen presenting functions.

In another embodiment, the immune cell is a NK cell or lymphokine activated cell (LAK), which upon expression of the chimeric TCR can recognize and specifically kill the desired target cell. A chimeric receptor based on the killing activating receptor (KAR) or killing inhibitory receptor (KIR) may have very important applications using NK cells. In another embodiment, the present invention provides a target cell antigen-specific immune cell, said immune cell containing an antigen-binding molecule chimerized to a signal transducing element. The antigen-binding molecule may be selected from the group consisting of, but not limited to, T cell receptors, T cell receptor fragments or any protein recognizing and binding to MHC-presented peptides. In a preferred version of this embodiment, the antigen-binding molecule is a chimeric T cell receptor. Most preferably, the chimeric T cell receptor is a 2D or 3D scFv or a tcFv chimeric TCR.

In the target cell antigen-specific immune cell of the present invention, the antigen-binding molecule binds to a mammalian cell in a MHC restricted fashion or a MHC non-restricted fashion. In the target cell antigen-specific immune cell, the signal transducing element is selected from the group consisting, but not limited to, CD2, CD3, CD16, CD28, γ-chains, ε-chains, ζ-chains, KIR, KAR and fragments thereof.

In the target cell antigen-specific immune cell of the present invention, the antigen-binding molecule binds to an antigen selected from the group consisting of viral antigens, synthetic antigens, tumor associated antigens, tumor specific antigens, mucosal antigens, superantigens, differentiation antigens, auto-immune antigens, class I major histocompatibility complex molecules, class II major histocompatibility complex molecules and epitopes of antigen-specific T cell receptor or antibodies.

It is specifically contemplated that pharmaceutical compositions may be prepared

using the novel immune cells of the present invention. A person having ordinary skill in this art would readily be able to determine, without undue experimentation, the appropriate dosages and routes of administration of the novel pharmaceutical composition of the present invention. Accordingly, the present invention also provides for a pharmaceutical composition comprising the immune cells of the invention. The pharmaceutical composition of the present invention may further comprise a pharmaceutically acceptable carrier as is well known in the art.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLES

MATERIALS AND METHODS

Cells and antibodies.

T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere (Wejtens et al.,1996). Target cell lines used in this study are: *(i)* the HLA-A1^{POS}, MAGE-1^{POS} melanoma cell line MZ2-MEL.3.0, (*ii*) the HLA-A1^{POS}, MAGE-1^{NEG} melanoma cell line MZ2-MEL 2.2 (kindly provided by T. Boon and P. Coulie, Brussels, Belgium), *(iii)* the HLA-A1^{POS}, MAGE-1^{POS} melanoma cell line 518A2 (kindly provided by P. Schrier, Leiden, the Netherlands), *(iv)* the HLA-A1^{POS}, MAGE-1^{NEG} melanoma cell line Mel 2A, (*v*), the HLA-A1^{NEG} melanoma cell line MEL 78 . *(vi)* the HLA-A1^{NEG} melanoma cell line BLM, (*vii*) the HLA-A1^{NEG} renal carcinoma cell line SKR17-4 and *(viii)* the HLA-A1^{POS} EBV transformed B cell blasts APD and 72-2 (kindly provided by P. Traversari, Milan, Italy) . The human embryonic kidney cell line 293T (kindly provided by Y. Soneoka, Oxford, UK; 20) was used as packaging cell line for the pSTITCH $\alpha\zeta/\beta\zeta$ and pBullet $V_\alpha V\beta C\beta\zeta$ retroviral vectors (Weijtens et al., 1998). The mouse packaging cell line PG13 (ATCC CRL-10686) was used to obtain stable full-length TCR $\alpha\beta$ retrovirus producing cells. The CTL clone MZ2-82/30 was used for isolation of RNA coding for the HLA-A1 restricted, MAGE-1 specific TCR (kindly provided by T. Boon and P. Coulie, Brussels, Belgium); The mAbs used in this study were: anti-HLA-ABC (clone W6/32, Sera-Lab, Crawley Down, UK),

anti-CD4^{CY-5}, anti-CD8$^{FITC/PE}$ (Becton Dickinson Biosciences, San Jose, USA) and the TCR V_α and V_β family specific mAb anti-V_α12.1 (T Cell Diagnostics, USA) and anti-V_β1 (Coulter-Immunotech, Marseille, France).

Retroviral (chimeric-) TCR gene transduction into primary human T lymphocytes: *in vitro* expansion of transduced T lymphocytes.

Activated human Peripheral Blood Lymphocytes (PBL) (5 x 10^6) were transduced using the pSTITCH or pBullet retroviral vector with the chimeric two chain TCR $\alpha\zeta/\beta\zeta$ genes or chimeric single chain TCR $V_\alpha V_\beta C_\beta\zeta$ respectively, as described earlier (Weijtens et al., 1998). Briefly, anti-CD3 activated lymphocytes were incubated for 72 hrs with an irradiated (25 Gy) monolayer of recombinant retrovirus-producing 293T cells, using culture medium (RPMI 1640 with 25 mM HEPES, 10% human serum, 2mM Glutamine, Penicillin 100 U/ml and streptomycin 100 μg/ml) supplemented with 4μg/ml polybrene (Sigma, St. Louis, U.S.A.), and 360 IU/ml rIL-2 (Proleukin; Chiron, Amsterdam, the Netherlands). Retroviral transduction with full-length TCR $\alpha\beta$ genes was performed with supernatant produced on PG13 packaging cells containing both pBullet TCR α and TCR β vectors essentially as described (Van de Griend et al., 1984). Expansion of transduced primary human T lymphocytes was performed in the presence of feeder cells as we described elsewhere (Van de Griend et al. 1984; Pollok et al., 1998).

Immunofluorescence analysis and sorting of retroviral transduced human T lymphocytes.

Cells (5 x 10^5) were stained with TCR V_α and V_β family-type specific anti-V_α-12.1^{FITC} (2 μg/ml) and anti-V_β-1^{PE} (2 μg/ml) mAb in a volume of 50 μl. The dotplots show viable T lymphoblasts selected by gating on forward (FSC) and sideward (SSC) light scatter signals. Analysis was performed on a FACSCAN instrument (Becton Dickinson Biosciences, San Jose, USA). Flow sorting was performed on a FACS-Vantage instrument (Becton Dickinson Biosciences) using saturating concentrations of anti-V_α-12.1^{FITC}, anti-V_β-1^{PE} or Multimeric HLA-A1/peptide/ streptavidin^{PE} complexes (1:10 dilution of fresh preparations).

Cytotoxicity assays.

Cytolytic activity of transduced T lymphocytes was measured in 6 hr ^{51}Cr-release assays

as described (Weijtens et al., 1998). In experiments aimed at blocking specific cytolytic activity, mAbs were added to the CTL, 15-30 min before addition of the target cells (W6/32: 10 μg/ml, or irrelevant mIg: 10 μg/ml). Peptide loading of the target cells was performed by addition of 10 μg/ml MAGE-1 nonapeptide (EADPTGHSY) or irrelevant Influenza peptide derived from Influenza virus A nucleoprotein (CTELKLSDY) to the target cells prior to incubation with effector T lymphocytes.

GM-CSF, TNF$_\alpha$ and IFN-γ production.

To quantify GM-CSF and TNF$_\alpha$ production by the gene-transduced T lymphocytes after antigen-specific stimulation, $6x10^4$ transduced PBL were cultured for 24 h either in the presence or absence of $2x10^4$ adherent tumor cells in RPMI-1640 medium supplemented with 360 IU/ml rIL-2. At the end of culture, supernatant was harvested and levels of TNF-α, GM-CSF (Medgenix, Fleurus, Belgium) and IFN-γ (CLB, Amsterdam, the Netherlands) were measured by standard ELISA according to the manufacturers instructions.

EXAMPLE 1

Isolation and cloning of a MAGE-1 specific (scFv)-TCR from the CD8$^+$ CTL clone 82/30.

Standard cloning techniques were used in the examples below. Techniques are described in: Molecular Cloning; A Laboratory Manual (Cold Spring Harbor Press, Cold Spring Harbor, N.Y.) by Maniatis, T. et al. or in cited publications.

Cloning of the TCR Vβ

The TCR is a heterodimer which consists of an α and a β chain. The TCR α and β chains are members of the immunoglobulin gene superfamily and are generated by combined associations of V, J, D and C genes. The TCR polypeptides are disulfide linked, highly polymorphic in their N-terminal, variable domains and responsible for antigen recognition. Obtaining the TCR from the MAGE-1 specific CTL clone 82/30 (Traversari, C. et al., 1992) was achieved by PCR (Maniatis, T. et al.) amplification of cDNA obtained from this CTL clone.

To obtain cDNA, total RNA was isolated from T cell clone 82/30 cells according to the method by Chomczynski et al. (Chomczynski, P. and Sacchi, N., 1987) and transferred into cDNA essentially as described by Maniatis et al. Amplification of cDNA

sequences by PCR is possible only if the sequence of the gene of interest is known. In general, for PCR, two primers complementary to the 5′ end and the 3′ end of the sequence are used as the initiation point of DNA synthesis. Because the sequence of the 5′ ends of the TCR α and β chain from T cell clone 82/30 were unknown, a PCR method, referred to as RACE (rapid amplification of cDNA ends) was used to amplify the TCR α chain (Edwards et al., 1991). The TCR β chain was amplified by RACE-PCR using primers described in Table 2.

Oligonucleotide primers used to synthesize the α and β chain cDNA and amplify the V_α, V_β and C_β gene segments (Table 2)

A fragment of about 350-450 base pairs was isolated from the agarose gel, purified and ligated into pBluescript (Stratagene, USA) as described by Maniatis et al. The ligation mixture was introduced into bacteria which were selected and expanded. DNA was isolated from these selected bacterial colonies and analyzed by restriction enzyme digestion to confirm the presence of the amplified TCR β fragment.

Three positive colonies were subjected to DNA sequencing. The sequences of these three individual clones were compared and found to be identical. The sequence obtained from the amplified TCR β fragments however did not include the signal sequence of the TCR β gene. To obtain the complete sequence of the TCR-V β gene, this partial sequence was compared with all known TCR β sequences. Alignment of the sequences showed almost 100% homology to sequences from the TCR β family 1 (TCRβ.1) (Hanspeter Saluz and Jean-Pierre Jost, 1989). Based on this sequence homology, a primer was synthesized complementary to the 5′ end of the TCR β.1 and used to amplify the complete TCR variable β.1

1987). Analysis of the obtained sequence showed 100% homology of the amplified TCR β fragment to the signal sequence and major part of the variable region of the TCR β chains from the TCR β family 1. Based on this sequence primers were designed that allowed cloning of the TCR variable β chains in a single chain TCR construct.

EXAMPLE 2

Cloning of the TCR α chain

For cloning of the TCR α chain, a different approach was followed. First, to determine to which family the TCR V_α chain belongs, a family typing PCR (Thor Straten,

P. et al. 1994) was performed. Twenty-one different TCR variable α chains have been described. In a family typing PCR, the template cDNA was divided into separate samples that are each individually amplified with a family specific 5′ primer and a constant primer. Multiple PCR reactions had yielded amplified fragments. In order to determine which fragment corresponded to the TCR $V\alpha$ fragment, a Southern blot was performed (Maniatis et al.) with a ^{32}P-labeled $C\alpha$ probe. A positive signal was observed only in the PCR reaction which was performed with the TCR α primer corresponding to family number 12. The rest of the DNA of this PCR reaction was purified with primer removal as described above and subjected to DNA sequencing. The obtained sequence was compared to the TCR α sequence of the only known member of family number 12 (Sim et al., 1984; Berkhout et al., 1998). Except for the diversity region, 100% homology was observed. This allowed the design of primers which could be used for the amplification of the complete variable region of the TCR α chain. The sequenceS of $V\alpha$ and $V\beta$ of the TCR derived from CTL clone 82/30 are shown in Figs. 2A and 2B, respectively.

EXAMPLE 3

Construction of chimeric two domain and three domain single chain TCR/γ or ζ molecules.

Construction of two domain single chain TCR/γ or ζ molecules.

For construction of chimeric single chain TCR/γ or ζ molecules, a cloning vector was designed that allows easy construction of single chain molecules. The vector was made by replacement of the multiple cloning sites in pBluescript (Stratagene) by a specially designed polylinker (Table 3). For cloning of the TCR $V\alpha$ and TCR $V\beta$ fragments, primers were designed that allowed cloning of these fragments in front of the flexible linker sequence or after the linker sequences (Fig. 2).

For amplification of the TCR $V\alpha$ and TCR $V\beta$ fragments, two separate PCR reactions were performed to generate fragments that include the signal sequence of the V region and fragments that start practically at the beginning of the mature protein. The DNA fragments were digested with restriction enzymes that allow cloning next to the flexible linker. Positive bacterial colonies were grown for DNA purification and DNA was subjected to DNA sequencing. DNA clones with the correct sequence were used to construct the chimeric single chain TCR constructs. The clones containing either the TCR $V\alpha$ or the TCR $V\beta$ fragments in front of the flexible linker sequence were then ligated to

the TCR $V\alpha$ and TCR $V\beta$ fragments which lack the signal sequence. In this way, three different single chain TCRs were constructed ($V\alpha$-212 linker-$V\beta$, $V\beta$-212 linker-$V\alpha$ and $V\alpha$-$(Gly_4Ser)_3$, linker-$V\beta$), see Figure 3.

For ligation of the two domain single chain TCR to γ or ζ molecules, Bam HI and Xho 1 digested human γ and ζ DNA fragments were ligated 3′ to the two single chain TCR constructs. The following chimeric two domain single chain TCR γ and ζ constructs were made: $V\alpha$-212 linker-β-γ $V\alpha$-212 linker-β-ζ, $V\alpha$-$(Gly_4Ser)_3$-$V\beta$-γ, $V\beta$-212 linker-$V\alpha$-γ and $V\beta$-212 linker $V\beta$-ζ.

Construction of three domain (3D) TCR/γ or ζ molecules

For the construction of chimeric three domain (3D) single chain TCR molecules the constant β chain domain was amplified by PCR from cDNA obtained from CTL 82/30 and inserted next to the chimeric single chain TCR $V\alpha$-212 linker-$V\beta$. The primers used to amplify the $C\beta$ region are described in Table 4. The 3D TCR $V\alpha$-212 linker-$V\beta$-$C\beta$ fragment was then reamplified by PCR with primers that introduced restriction sites allowing cloning into a retroviral pBullet expression cassette. The primers used for reamplification are described in Table 5.

Construction of chimeric two chain TCR/ζ molecules

For the construction of chimeric two chain TCR/ζ molecules, RNA isolated from CTL clone 82/30 was amplified using $V\alpha$-ATG and $C\alpha$-cys primers and $V\beta$ and $C\beta$-cys primers respectively. (Table 5). To construct the $V\alpha C\zeta$ and $V\beta C\beta\zeta$ genes ($\alpha\zeta\beta\zeta$), $V\alpha C\alpha$ and $V\beta C\beta$ were each ligated 5′ to the ζ gene in pBluescript. Correct sequences of chimeric $V\alpha C\alpha$ and $V\beta C\beta$ constructs were then cloned into the pSTITCH retroviral vector.

EXAMPLE 4

Cloning of the two domain (scFv)-TCR-γ into the retroviral vector SFG and generation of stable virus producing packaging cells.

The chimeric single chain $V\beta$-212 linker-$V\alpha$–γ construct was ligated into the SFG retroviral vector (Figure 4) by standard cloning techniques.

For the generation of virus producing amphotropic packaging cell lines the SFG-$V\beta$-212-$V\beta$-γ retroviral vector was electroplated into the amphotropic packaging cell line PA317, using the BTX electro cell manipulator 600 (settings: 760 uF, 250 V, 129 Ω). PA317 cells expressing the $V\beta$-212-$V\beta$-γ chain were selected by a monoclonal antibody

directed against the V_α chain (Diversi-T^{tm} V_α12, T cell Diagnostics, Cambridge, USA) bound to goat anti-mouse coated magnetic particles. The packaging cells containing the SFG-Vβ-212-V$_{\alpha-\gamma}$ construct were used to transduce anti-CD3 activated human PBL.

Cloning the chimeric three-domain scFv TCR γ or ζ into the retroviral pBullet cassette.

For expression of chimeric three domain single chain Ig/γ or ζ and TCR/γ or ζ molecules in human T lymphocytes, the retroviral vector pBullet_was constructed. After removal of two Nco I sites (positions 316 and 3952) and the Xho I site (position 3834), in the retroviral vector pSTITCH (Weijtens, M.E.M., et al. 1998) a multiple restriction site sequence was introduced. The resulting vector was named pBullet. Into the Nco I site at position 1968 and the newly introduced Xho I site of pBullet an expression cassette sequence was introduced containing the signal sequence from the G250 mAb Variable heavy chain, two cloning sites for introduction of the three domain single chain TCR and a signal transducing element. Two signal transducing elements have been introduced: one containing a short constant Ig kappa chain sequence Ck, extracellular domain of ζ, the CD4 transmembrane domain and the intracellular domain of the γ chain. The other signal-transducing element comprises the complete ζ, chain, starting at the Bam H I site present in ζ. The chimeric three domain TCR construct which were reamplified with primers shown in Table 5, were cloned into the pBullet vector containing the expression cassette. Sequences of the complete chimeric 3DTCR/CkCD4Tm$_\gamma$ and 3DTCR/ζ, were verified.

Generation of stable packaging cell lines containing the three domain TCR/γ or ζ constructs.

For the generation of stable packaging cell lines producing the three domain TCR/γ or ζ viruses PG13 packaging cells were cocultivated for two days with irradiated 293T cells (25 Gray), transfected with a: the retroviral vector pBullet 3D/CkCD4Tm$_\gamma$ or pBullet 3D/ζ b: the amphotropic envelope construct pHIT 456 and c: the GAG-POL construct pHIT 60. PG13 packaging cells expressing the three domain TCR$_\gamma$ or ζ construct, determined by flow cytometry using anti- V_α12.1 and anti-Vβ1 mAb, were selected by flow cytometric sorting. The efficiency of retrovirus production by PG13 packaging cell lines PG13-3DCkCD4Tm$_\gamma$ and PG13-3Dζ was determined by applying serial dilutions of viral supernatant to 293T cells, which were analyzed two days later

for the percentage of $V_\alpha 12.1^{pos}$, $V\beta 1^{pos}$ cells. Stable high producer PG13 packaging cells were used to produce viral supernatant used to transduce anti-CD3 activated human PBL.

EXAMPLE 5

Infection and expression of the chimeric (scFv)-TCR$_\gamma$ or ζ, in T cells.

To obtain optimal numbers of dividing T lymphocytes and optimal gene transduction, fresh or cryopreserved/thawed peripheral blood mononuclear cells (PBMC) from healthy donors were activated using anti-CD3 monoclonal antibody (mAb) and recombinant interleukin-2 (IL-2). Retroviral transduction of chimeric TCR $\alpha\zeta/\beta\zeta$ gene constructs and chimeric (scFv)-TCR$_\gamma/\zeta$ gene constructs was performed by incubating human T lymphocytes with 293T packaging cells that were transiently transfected with: (*i*) two retroviral vectors each comprising chimeric TCR $\alpha\beta$ gene constructs; (*ii*) a vector containing the retroviral Gibon Ape Leukemia virus envelope and (iii) a vector containing the viral GAG and POL genes. The full-length TCR α and β genes were introduced into human T lymphocytes derived from a different donor by incubation with supernatant obtained from PG13 packaging cells that contain both full-length TCR α and β genes.

Flow cytometric analysis of gene-transduced T lymphocytes demonstrated expression of the chimeric-TCR $\alpha\zeta/\beta\zeta$ and chimeric 3D (scFv)-TCR$_\gamma$ or ζ transgenes in 25 to 40% of primary activated, human T lymphocytes as evidenced by the strictly coordinated reactivity with anti-TCR V_α-12.1 and anti-Vβ-1 family typing mAbs respectively Fig. 5 shows the expression of (*i*) chimeric single chain TCR $V_\alpha V\beta C\beta\zeta$, (*ii*) chimeric two chain $V_\alpha C\alpha\zeta$ /$V\beta C\beta\zeta$, and (*iii*) full-length TCR $\alpha\beta$ constructs which was determined by flow cytometry after gene transduction of primary human T lymphocytes. Events shown represent viable lymphocytes stained with anti-$V_\alpha 12.1^{FITC}$ and anti-$V\beta 1^{PE}$. **(A):** Human T lymphocytes transduced with the CD24 gene (negative control). **(B):** HLA-A1 restricted, MAGE-1 specific parental CTL clone MZ2-82/30 (positive control). **(C):** Human T lymphocytes transduced with $V_\alpha V\beta C\beta$-ζ retroviral vectors **(D)**: Human T lymphocytes transduced with $V_\alpha V\beta C\beta$-ζ retroviral vectors and enriched by flow sorting using anti-$V_\alpha 12.1$ FITC and anti-Vβ-1 PE mAbs. **(E)**: Human T lymphocytes transduced with $V_\alpha C_\alpha$-ζ and $V\beta C\beta$-ζ retroviral vectors simultaneously. **(F)**: Human T lymphocytes transduced with $V_\alpha C_\alpha$-ζ and $V\beta C\beta$-ζ retroviral vectors and

followed by enrichment by flow sorting using anti-V_α12.1 FITC and anti-$V\beta$-1 PE mAb. **(G)**: Human T lymphocytes transduced with full-length TCR α and β retroviral vectors simultaneously. **(H)**: Human T lymphocytes transduced with full-length TCR α and β retroviral vectors, followed by V_α12.1 immunomagnetic selection and a second enrichment by flow sorting using soluble multimeric HLA-A1/MAGE-1/Strep^{PE} complexes. FACS analysis using anti-V_α12.1 and anti $V\beta$1 show identical fluorescence signals as the 3D scFv TCR $V_\alpha V\beta C\beta\zeta$.

EXAMPLE 6

(Chimeric) (scFv)-TCR$_\gamma$ or ζ^{POS} T lymphocytes bind soluble multimeric HLA-A1/MAGE-1 complexes.

The HLA-A1 binding MAGE-1 peptide EADPTGHSY and an irrelevant HLA-A1 binding peptide derived from Influenza virus A nucleoprotein (CTELKLSDY) were used to construct soluble peptide-MHC complexes for the identification of antigen specific TCR on gene transduced T lymphocytes. Tetrameric (or multimeric) complexes comprising soluble MHC class I molecules with antigenic peptides and PE conjugated streptavidin are reagents that allow the identification of MHC restricted, antigen specific T lymphocytes. Binding of tetrameric complexes has been shown to correlate with recognition of viral antigens presented by virally infected cells or tumor associated antigens presented on tumor cells by cytotoxic T cells (Cole et al., 1995). Tetramer binding T cells were shown to recognize and kill virally infected cells or tumor cells.

Construction of multimeric HLA-A1/MAGE-1 peptide and HLA-A1/Influenza virus A nucleoprotein peptide complexes:

First the HLA-A1 heavy chain was cloned by the following protocol:

The gene coding for the HLA-A101 gene was amplified by PCR using the primers GCGGCGGCGGCCATGGGCTCCCACTCCATGAGG and TTTCTGTGCATCCAGAA TATGATGCAGGGATCCGAGCTCCCATCTCAGGGT, and the product of this first PCR was used as template in a second PCR using the primers GCGGCGGCGGCCATGGGCTCCCACTCCATGAGG and CGGCAGGAGA GCGGCCGCTTAACGATGATTCCACACCATTTTCTGTGCATCCAGAAT. The restriction sites *NcoI* and *NotI* used for cloning are underlined. The forward primers encode the peptide HHILDAQKMVWNHR recognized by the BirA enzyme used for in vivo biotinylation (see below). The PCR products were ethanol-precipitated, digested with *NcoI*

and *NotI* enzymes, gel-purified and ligated into the plasmid pET21d (Novagen, Madison,USA) digested with the same enzymes. The plasmids were transformed into DH5α and clones containing an insert were sequenced. Clones with the correct sequence were then transformed into BL21DE3 for protein production, together with a compatible plasmid containing the Bir A gene under the control of the tac promoter (pBirCm, Avidity™, denvers, USA). The plasmid pHNβ2m was used to produce the β2m.

Next, the HLA-A1 protein and β2m protein were purified by inclusion body purification:

The inclusion body purification of HLA-A1 proteins was done by a 10-ml overnight bacterial culture was diluted in one liter of 2xTY containing 100 μg/ml ampicillin and 15 μg/ml chloramphenicol and incubated at 37∘C with agitation. At OD600 of 0.5, 1mM IPTG and 50 μM biotin was added and the bacterial culture was incubated at 37∘C for 3-4 h. The bacterial culture was centrifuged and the pellet was resuspended in 20 ml of lysis buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-Hcl pH 8). 200 μl of 100 mM PMSF in isopropanol and 1 ml of 10 μg/ml lysozyme were added and the bacteria were incubated 30 min on ice. 200 μl of 1M MgCl2, 20 μl of 1M MnCl2 and 20 μl of 10 μg/ml DNAse1 were added and the mix was incubated again 20 min on ice. After sonication and centrifugation 15 min at 10 000 rpm at 4∘C, the bacterial pellet was resuspended in 20 ml of detergent buffer (0.2 M Nacl, 1% DOC, 1% Nonindet P40, 2 mM EDTA, 20 mM Tris 7.5). 40 μl of 1M DTT and 200 μl of 100 mM PMSF were added. After centrifugation (15 min 10000 rpm at 4∘C) the pellet was resuspended by sonication and centrifuged again, and then resuspended in 12 ml of triton buffer (100 mM NaCl, 50 mM Tris-Hcl pH 8, 1 mM EDTA, 0.5% Triton X100) plus 24 μl of 1M DTT. After centrifugation (15 min 10000 rpm at 4°C) the pellet was washed 2 more times in this buffer and finally resuspended in 2 ml of freezing buffer (20 mM Tris-Hcl pH 7.5, 150 mM NaCl, 10 mM DTT) and kept at -70∘C.

Finally the HLA-A1/peptide complexes were reconstituted by dilution:

For reconstitution of HLA-A1/peptide complexes, the inclusion bodies were thawed, centrifuged for 10 min at 10 000 rpm, and the protein pellet was dissolved in the same volume of urea buffer (50% urea, 50 mM NaCl, 20 mM Tris 7.5, 5 mM reduced glutathion). After a 10 min centrifugation at 10,000 rpm, the supernatant was used for refolding.The complex formation was initiated by dilution of the two denatured subunits and peptide into 200 ml of refolding buffer (400 mM Arginine/Hcl, 2 mM EDTA, 5 mM

reduced glutathion, 0.5 mM oxidized glutation, 100 mM Tris-Hcl pH 7.5). The amount of heavy chain, β2m and peptide were respectively 6 mg, 5 mg and 2 mg. The solution was incubated without agitation for 36h at 4◦C and then concentrated with Centriprep-10 from 200 ml to 700 μl. The concentrated protein was subjected to gel filtration on a Superdex 200 column (Pharmacia, Uppsala, Sweden) and the peak corresponded to 48 kDa was collected.

Multimeric HLA-A1/peptide/streptavidin^{PE} complexes used for staining of transduced lymphocytes were made by mixing equal volumes of streptavidin PE (5 μg/ml, Becton Dickinson Biosciences, San Jose, USA) and soluble HLA-A1/peptide (120 μg/ml), followed by 30 min incubation on ice. Chimeric (scFv)-TCR$_{\gamma/\zeta}$, were incubated for 30 min with 1:10 dilution of a fresh preparation of multimeric HLA-A1/MAGE-1/Streptavidin^{PE} complexes or HLA-A1/Influenza/Streptavidin^{PE} complexes at 4°C

As shown in figure 6, MAGE-1/HLA-A1/streptavidin^{PE} multimers specifically stained the genetically introduced chimeric two chain-αζ/βζ , chimeric scFv TCR/ζ and full-length αβ TCRs expressed on human T lymphocytes, but not control multimeric HLA-A1complexes presenting the Influenza peptide. Neither did control T lymphocytes, that were transduced with the enhanced green fluoresence protein (EGFP) transgene, recognize and bind soluble multimeric HLA-A1/MAGE-1 complexes. FACS analysis using multimeric HLA-A1/MAGE-1 complexes show identical fluorescence signals as the 3D scFv TCR $V_\alpha V\beta C\beta\zeta$.

EXAMPLE 7

Functional analysis of chimeric (scFv) TCR$_{\gamma/\zeta}^{POS}$ T lymphocytes.

MAGE-1 peptide loaded target cells specifically trigger lysis by *(i) chimeric 3D scFv-, (ii) chimeric tc- and (iii) full length-TCR^{POS}* T lymphocytes.

To determine whether chimeric *3D scFv-, tc- and full length-TCR^{POS}* T lymphocytes were able to recognize the MAGE-1 peptide, 51 Cr labeled MAGE-1^{NEG}, HLA-A1^{POS} melanoma cells (MEL 2A) and EBV transformed B cell blasts (72-2 and APD) were pulsed with 10 μg/ml MAGE-1 peptide or irrelevant Influenza virus peptide derived from Influenza virus A nucleoprotein. and incubated for 6 hour with the chimeric (scFv)-TCR^{POS} T lymphocytes T lymphocytes expressing chimeric *3D*

scFv-TCRs , chimeric *tc-TCRs* and full-length αβ TCRs were indeed able to lyse the MAGE-1 peptide loaded, MAGE-1^{NEG}/ HLA-A1^{POS} MEL 2A melanoma cells and the MAGE-1^{NEG}/ HLA-A1^{POS} B-lymphoblastoid cell lines 72-2 and APD. Only MAGE-1 peptide-pulsed MAGE-1^{NEG}/ HLA-A1^{POS} target cells were specifically lysed by the transduced T lymphocytes, but not the unloaded cells or cells loaded with the irrelevant Influenza peptide (Fig 7). Cytotoxicity analysis show that HLA-A1^{POS}, MAGE-1^{POS} melanoma cells are lysed by the 3D scFv TCR $V_\alpha V_\beta C_\beta CCkCD4Tm_\gamma^{POS}$ T lymphocytes as efficiently as by the 3D scFv TCR $V_\alpha V_\beta C_\beta \zeta^{POS}$ T lymphocytes.

HLA-A1^{POS}, MAGE-1^{POS} melanoma cells specifically trigger lysis by chimeric (scFv)-TCR^{POS} T lymphocytes

Several ^{51}Cr labeled HLA-A1^{POS} /MAGE-1^{POS} melanoma cell lines and control HLA-A1^{NEG} cell lines were incubated with the *chimeric 3D (scFv)-TCR^{POS}, tc-TCR^{POS} and full-length TCR^{POS}* T lymphocytes to determine the HLA-A1 restricted, MAGE-1 specificity of the transduced T lymphocytes. As shown in Fig.8 sorted chimeric *3D scFv-TCR^{POS}, tc-TCR^{POS}* and sorted-full-length TCR^{POS} T lymphocytes specifically lysed the HLA-A1^{POS}, MAGE-1^{POS} melanoma cells MZ2-MEL 3.0 and 518A2, but not the HLA-A1^{NEG} control cell lines (MEL 78, BLM and SKRC17-4) in a 6 hour incubation period. Further proof of MAGE-1 specificity of transduced T lymphocytes came from the addition of anti-MHC class I mAb, which resulted in significant lower tumor cell lysis, whereas irrelevant mAb did not inhibit cytolytic activity (Fig 8).

Analysis of lymphokine production by chimeric (scFv)-TCRγ/ζ^{POS} T lymphocytes.

HLA-A1 restricted, MAGE-1 specificity of the chimeric *3D scFv-TCR^{POS}, tc-TCR^{POS} and full length TCR^{POS}* T lymphocytes was analyzed by incubating these cells with MAGE-1 peptide pulsed HLA-A1^{POS}, MAGE-1^{NEG} Melanoma cells MZ2-MEL 2.2 and HLA-A1^{POS}, MAGE-1^{POS} melanoma cells MZ2-MEL 3.0. After 24 hour incubation supernatant was harvested and analyzed for the presence of TNF_α, GM-CSF and IFN_γ. Table 6 shows the results of the incubation of the CTL with HLA-A1^{POS}, MAGE-1^{POS} melanoma cells and MAGE-1 peptide pulsed melanoma cells significant amounts of these lymphokines were produced.

Soluble multimeric HLA-A1/MAGE-1 complexes identify functional *(i) chimeric 3D scFv-TCR, (ii) chimeric tc-TCR and (iii) full length TCR* expressing T lymphocytes.

Soluble mulimeric HLA-A1/MAGE-1/streptavidin complexes could be used to identify and select peptide specific cells from bulk cultured gene transduced T lymphocytes. Therefore, chimeric 3D scFv-TCR, chimeric tc-TCR and full-length TCR $\alpha\beta$ gene transduced T lymphocytes were sorted with soluble multimeric HLA-A1/MAGE-1/streptavidin^{PE} complexes. Positive and negative fractions obtained by sorting were expanded and analyzed for HLA-A1 restricted, MAGE-1 specificity.As shown in figure 9, CD8^{POS} chimeric *3D scFv-TCR, chimeric tc-TCR* and full-length TCR $\alpha\beta$ gene transduced T lymphocytes enriched by soluble multimeric HLA-A1/MAGE-1/streptavidin^{PE} complexes specifically lysed not only MAGE-1 peptide pulsed B-LCL target cells but also by the HLA-A1^{POS}, MAGE-1^{POS} MZ2-MEL 3.0 melanoma cells. The negative fraction obtained from these sorts; were not able to lyse the HLA-A1^{POS}, MAGE-1^{POS} melanoma cells and only a weak response to peptide pulsed target cells was observed. Surprisingly, both the negatively and positively sorted Full-length TCR $\alpha\beta$ gene transduced T lymphocytes express the introduced TCR $\alpha\beta$ as determined by flow cytometry using family typing mAbs anti-Vα12.1^{FITC} and anti-Vβ1^{PE}.

Table 1:

Antigens on Autologous Human Cancers Recognized by T Cell Responses

Antigen	Tumor Type	Normal Tissue Expression	Comments
MAGE-1	Melanoma, lung	Testes	Not expressed in melanocytes
MAGE-3	Melanoma, lung	Testes	Not expressed in melanocytes
Tyrosinase	Melanoma	Melanocytes	Melanosomal protein
Albino			
MUCI	Pancreas, breast	Epithelium	Non-MHC restricted
Melan-A/	Melanoma	Melanocytes	Melanosomal protein
MART-1		and retina	
PMe117/silver	Melanoma	Melanocytes	Melanosomal protein
		and retina	
GP100	Melanoma	Melanocytes	

Table 2.:

Oligonucleotide Primers

V_α-ATG: 5′ GCG AAT TCT ACG TAC CAT GAA CAT GCT GAC TGC CAG C3′

V_α-3 5′ CGT CTA GAG GAC AGA AGG TAA CTC AAG CGC AG 3′

V_β-ATG: 5′ CCG AAT TCT ACG TAC CAT GGG CTT CAG GCT GCT CTG 3′

V_β-3 5′ GCG GAT CCG AGC ACT GTC AGC CGG GTG CC 3′

Table 3:

212 LINKER

5′ GTA CGA ATT CGC AGA TCT GGC TCT ACT TCC GGT AGC AAA TCC TCT GAA GGC AAA GGT ACT AGT GCG GAT CCG GCT CGA GCA GCT 3′

$(GLY)_4SER_3$ LINKER

5′ GAT CCG GTG GAG GCG GTT CAG GCG GAG GTG GCT CTG GCG GTG GCG GAT CGA 3′

Table 4: Primers used for amplification of the TCR β constant region and for reamplification of the three domain TCR V_α-212 linker-$V_\beta C_\beta$ fragment.

C_β 5′ primer: 5 -CTG-ACA-GTG-CTC-GAG-GAC-CTG-3′

C_β 3′ primer: 5 -CTC-TGG-ATC-CCG-TCT-GCT-CTA-CCC-CAG-GGG-TG-3′

V_α 5′ primer for reamplification: 5′-GCG-AAT-TCT-ACG-TAC-CAT-GGA-CAT-GCT-GA-TGC-CAG-C-3′

C_β primer for reamplification: 5 -TTG-TTC-TGC-GGC-CGC-GTC-T′′GC-TCT-ACC-CCA-GGC-3′

Table 5: Primers for reamplification of the three domain TCR fragment allowing introduction into the pBullet cassette vector.

TCR V_α 5′ primer: 5′-TTA-CTC-GCG-GCC-CAG-CCG-GCC-ATG-GCC-CAG-AAG-GTA-ACT-CAA-GCG-CAG-3′

TCR V_β 3 5′-TTG-TTC-TGC-GGC-CGC-GAG-CAC-TGT-CAG-GGT-GCC-TG-3′

Table 6: Chimeric TCR expressing T cells undergo specific stimulation to produce cytokines

A	MAGE-1 peptide pulsed melanoma cells *					
	TNFα pg/ml		GM-CSFpg/ml		IFNγ pg/ml	
	No pept.	+ MAGE-1 pept.	No pept.	+MAGE-1 pept.	No pept.	+ MAGE-1 pept
CTL 82/30	0.0	2996	0.0	426	39	6678
ch-scTCR VαVβCβζ^{POS} T lymph.	56	105	0.0	629	34	577
ch-2c αζ/βζ^{POS} T lymph.	61	380	0.0	3767	97	1954
full-length αβ^{POS} T lymph.	4.0	75	0.0	1924	91	1024

B	Native HLA-A1^{POS}/MAGE-1^{POS} melanoma cells *		
	TNFα pg/ml	GM-CSF pg/ml	IFNγ pg/ml
CTL 82/30	428	54	1159
ch-scTCR VαVβCβζ^{POS} T lymph.	85	1889	272
ch-2c αζ/βζ^{POS} T lymph.	120	1785	306
full-length αβ^{POS} T lymph.	15	832	211

* As described in materials and methods

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CLAIMS

1. An immune cell having predefined specificity, wherein said immune cell is either complexed with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) or a fragment thereof, or transfected with an antigen-specific MHC-restricted chimeric TCR gene.

2. The immune cell according to claim 1, wherein said chimeric TCR comprises: (i) a first segment comprising either (a) a single-chain TCR (scFv-TCR) made of the variable (V) region and, optionally, of either the extracellular constant (C) region of an antigen-specific TCR, or of the constant region of the immunoglobulin kappa light chain (Ck); or (b) a two-chain TCR (tcFv-TCR) made of the extracellular variable (V) and constant (C) regions of an antigen-specific TCR; and (ii) a second segment comprising a signal transducing element of an immune cell.

3. The immune cell of claim 2, wherein said scFv-TCR or tcFv-TCR comprise the alpha and beta chains pair or the gamma and delta chain pair of an antigen-specific TCR.

4. The immune cell according to claim 2 or 3, wherein said single-chain TCR of the first segment comprises the variable (V) region of said antigen-specific TCR.

5. The immune cell according to claim 4, wherein said single-chain TCR of the first segment is a two-domain (2D) single-chain TCR made of the extracellular variable (V) region $V\alpha V\beta$ chains of said antigen-specific TCR linked by a linker.

6. The immune cell according to claim 4, wherein said single-chain TCR of the first segment is a three-domain (3D) single-chain TCR made of the extracellular variable (V) and constant (C) regions of said antigen-specific TCR.

7. The immune cell according to claim 5, wherein said three-domain (3D) single-chain TCR comprises the $V\alpha V\beta C\beta$ or $V\alpha V\beta C\alpha$ chains of said antigen-specific TCR.

8. The immune cell according to claim 4, wherein said single-chain TCR of the first segment is a three-domain (3D) single-chain TCR made of the extracellular variable (V) $V\alpha V\beta$ chains of said antigen-specific TCR and the constant (C) region of the immunoglobulin kappa light chain (Ck).

9. The immune cell according to claim 2 or 3, wherein said first segment comprises a two-chain TCR (tcFv-TCR) made of the extracellular variable (V) and constant (C) regions of said antigen-specific TCR.

10. The immune cell according to claim 9, wherein said two-chain TCR (tcFv-TCR) comprises the $V\alpha C\alpha$ + $V\beta C\beta$ chains of said antigen-specific TCR.

11. The immune cell according to any one of claims 1-6, wherein said second segment comprises a signal transducing element of an immune cell comprising the intracellular signaling unit alone or together with the transmembrane domain and optionally with the extracellular domain of signaling chains.

12. The immune cell according to claim 11, wherein said signal transducing element is selected from the group comprising the TCR/CD3 complex gamma, delta, epsilon, eta or zeta chain, the gamma chain of the Fc receptor, the alpha, beta and/or gamma subunit of the IL-2 receptor, the CD2, CD16 and CD28 co-receptor chains, the signaling elements of the natural killer (NK) receptor, killing inhibitory receptor (KIR) or killing activating receptor (KAR), or any signaling element derived from an immune cell, and combinations of said signal transducing elements.

13. The immune cell according to claim 12, wherein said signal transducing element is selected from the group comprising the TCR/CD3 complex zeta chain or the gamma chain of the Fc receptor.

14. The immune cell according to any one of claims 1-13, wherein said immune cell is selected from the group comprising resting, activating and memory T lymphocytes, cytotoxic lymphocytes (CTLs), helper T cells , non-T lymphocytes, B cells, plasma cells, natural killer (NK) cells, monocytes, macrophages, eosinophils and dendritic cells.

15. The immune cell according to any one of claims 1-13, wherein said immune cell is selected from the group consisting of antigen-specific immune cells and non-specific immune cells.

16. The immune cell according to any one of claims 1-13, wherein said immune cell is an antigen-processing cell and said predefined specificity is targeted antigen uptake and presentation.

17. The immune cell according to any one of claims 1-13, wherein said immune cell is selected from the group consisting of lymphocytes, NK cells, macrophages, monocytes, eosinophils and other cytotoxic cells and said predefined specificity is target cell cytotoxicity.

18. The immune cell according to any one of claims 1-13, wherein said immune cell is a helper T cell and said predefined biological specificity is increased lymphokine or cytokine production.

19. The immune cell according to any one of claims 1-13, wherein said immune cell is selected from the group consisting of B lymphocytes and plasma cells and said predefined specificity is increased immunoglobulin production.

20. The immune cell according to any one of claims 1-13, wherein said immune cell is selected from the group consisting of T cells, B cells, monocytes and macrophages and said predefined biological specificity is increased antigen-presenting functions.

21. The immune cell according to any one of claims 1-13, wherein said immune cell is a target cell antigen-specific immune cell containing an antigen-binding molecule selected from the group consisting of the alpha/beta chains or the gamma/delta chains of the antigen-specific T cell receptor, chimerized to a signal transducing element selected from the group comprising the TCR/CD3 complex gamma, delta, epsilon, eta or zeta chain, the gamma chain of the Fc receptor, the alpha, beta and/or gamma subunit of the IL-2 receptor, the CD2, CD16 and CD28 co-receptor chains, the signaling elements of the

natural killer (NK) receptor, killing inhibitory receptor (KIR) or killing activating receptor (KAR), or any signaling element derived from an immune cell, and combinations of said signal transducing elements.

22. The target cell antigen-specific immune cell of claim 21, wherein said antigen-binding molecule binds to an antigen selected from the group consisting of viral antigens, synthetic antigens, tumor associated antigens, tumor specific antigens, mucosal antigens, superantigens, differentiation antigens, auto-immune antigens, class I MHC molecules and class II MHC molecules, and self antigens.

23. The target cell antigen-specific immune cell of claim 22, wherein said antigen-binding molecule binds to the melanoma-associated neoplastic protein (MAGE-1) antigen.

24. An immune cell complex according to any one of claims 1-23, wherein the chimeric antigen-specific TCR or TCR fragment is either bound to the immune cell by chemical conjugation or is bridged to the immune cell by a macromolecule or by a bispecific antibody binding both to the TCR or TCR fragment and to the immune cell.

25. The immune cell complex according to claim 24, wherein the macromolecule is avidin, streptavidin or polylysine.

26. The immune cell according to any one of claims 1-23, wherein said immune cell is transfected with said antigen-specific chimeric TCR gene.

27. A pharmaceutical composition comprising immune cells according to any one of claims 1-26.

28. The pharmaceutical composition according to claim 27, further comprising a pharmaceutically acceptable carrier.

29. The pharmaceutical composition according to claim 27 or 28 for the treatment of cancer, infectious diseases, autoimmune diseases or graft rejection.

30. The pharmaceutical composition according to claim 29 comprising the immune cells of claim 23 for the treatment of melanoma.

31. A method for treatment of a tumor in a patient comprising complexing lymphocyte cells of the patient with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) or a fragment thereof, or transfecting said autologous lymphocytes with an antigen-specific MHC-restricted chimeric TCR gene encoding a scFv-TCR which binds to an antigen associated with said tumor and a segment encoding a signal transducing element of an immune cell.

32. A method for treatment of melanoma in a patient comprising transfecting lymphocyte cells of the patient with an antigen-specific MHC-restricted chimeric T cell receptor (TCR) gene containing a segment encoding a scFv-TCR which binds to the melanoma-associated neoplastic protein (MAGE-1) antigen and a segment encoding a signal transducing element of an immune cell.

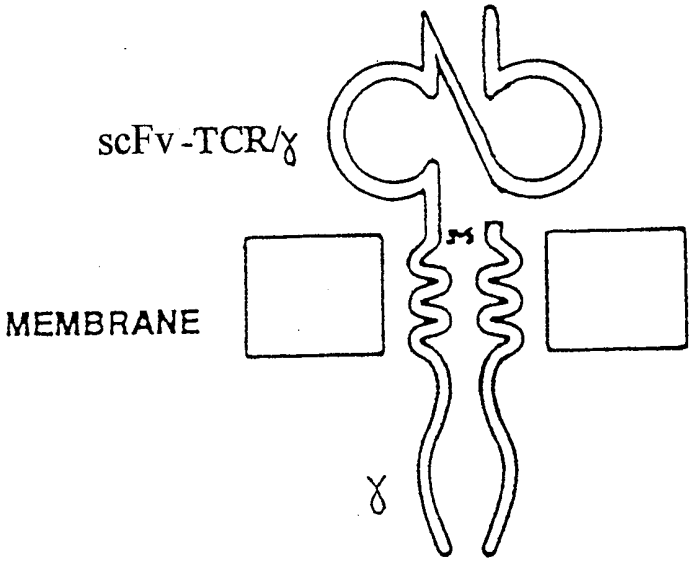
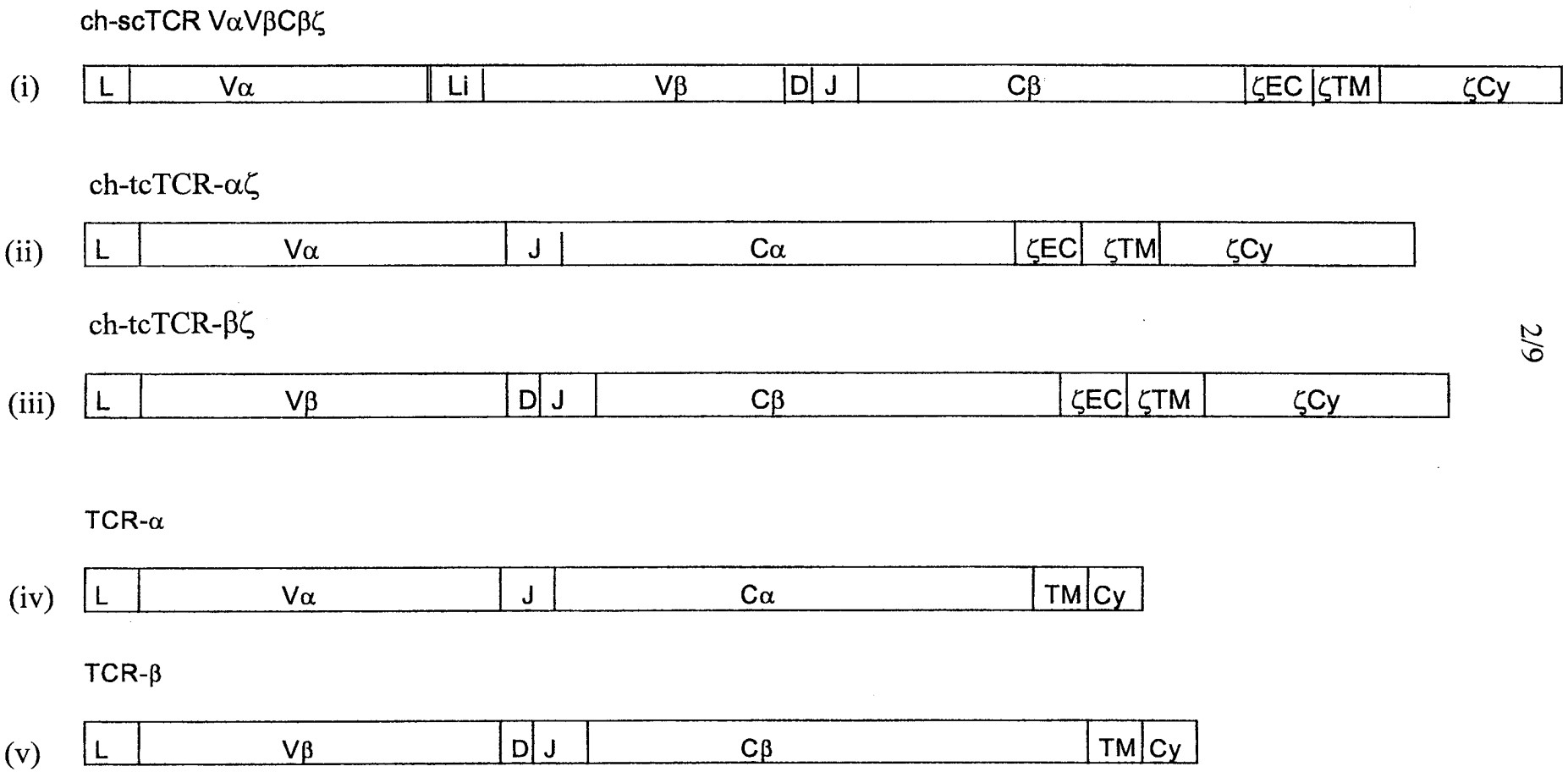


Fig. 1A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(iii)
L
Vβ
D
J
Cβ
ζEC
ζTM
ζCy
TCR-α
(iv)
L
Vα
J
Cα
TM
Cy
TCR-β
(v)
L
Vβ
D
J
Cβ
TM
Cy

Fig. 1B

Fig. 2A

Sequence of Vα:

ATG AAC ATG CTG ACT GCC AGC CTG TTG AGG GCA GTC ATA GCC TCC ATC
TGT GTT GTA TCC AGC ATG GCT CAG AAG GTA ACT CAA GCG CAG ACT GAA
ATT TCT GTG GTG GAG AAG GAG GAT GTG ACC TTG GAC TGT GTG TAT GAA
ACC CGT GAT ACT ACT TAT TAC TTA TTC TGG TAC AAG CAA CCA CCA AGT
GGA GAA TTG GTT TTC CTT ATT CGT CGG AAC TCT TTT GAT GAG CAA AAT
GAA ATA AGT GGT CGG TAT TCT TGG AAC TTC CAG AAA TCC ACC AGT TCC
TTC AAC TTC ACC ATC ACA GCC TCA CAA GTC GTG GAC TCA GCA GTA TAC
TTC TGT GCT CTG GGA GGG GTG AAT AAT AAT GCA GGC AAC ATG CTC ACC
TTT GGA GGG GGA ACA AGG TTA ATG GTC AAA CCC

Fig. 2B

Sequence of Vß:

ATG GGC TTC AGG CTG CTC TGC TGT GTG GCC TTT TGT CTC CTG GGA GCA
GGC CCA GTG GAT TCT GGA GTC ACA CAA ACC CCA AAG CAC CTG ATC ACA
GCA ACT GGA CAG CGA GTG ACG CTG AGA TGC TCC CCT AGG TCT GGA GAC
CTC TCT GTG TAC TGG TAC CAA CAG AGC CTG GAC CAG GGC CTC CAG TTC
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GAA CGA TTC TCC GCA CAA CAG TTC CCT GAC TTG CAC TCT GAA CTA AAC
CTG AGC TCT CTG GAG CTG GGG GAC TCA GCT TTG TAT TTC TGT GCC AGC
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Fig. 2C

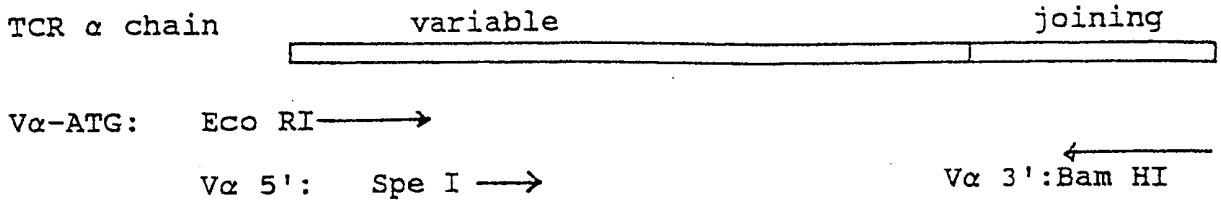
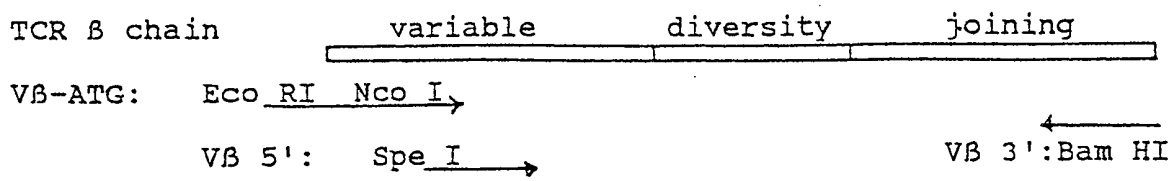


Fig. 2D



Vβ-212-Vα:

pBluescript	Eco RI	Nco I-Vβ-212-Vα-Bam HI	Xho I	pBluescript

Vα-212-Vβ:

pBluescript	Eco RI-Vα-212-Vβ-Bam HI	Xho I	pBluescript

Vβ-$(Gly)_4Ser_3$-Vα:

pBluescript	Eco RI	Nco I-Vβ-$(Gly)_4Ser_3$-Vα-Bam HI	Xho I	pBluescript

Fig. 3

A: SFG Vβ-212-Vα-γ

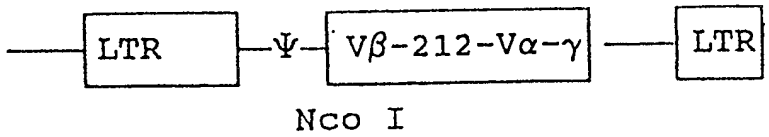


Fig. 4A

B: LXSN Vα-212-Vβ-γ

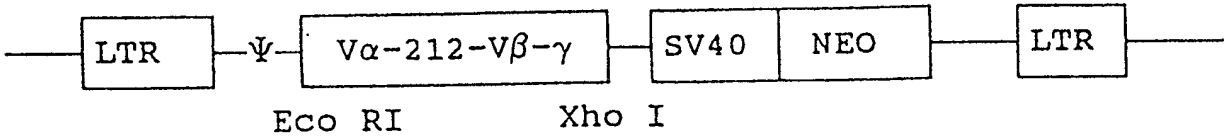


Fig. 4B

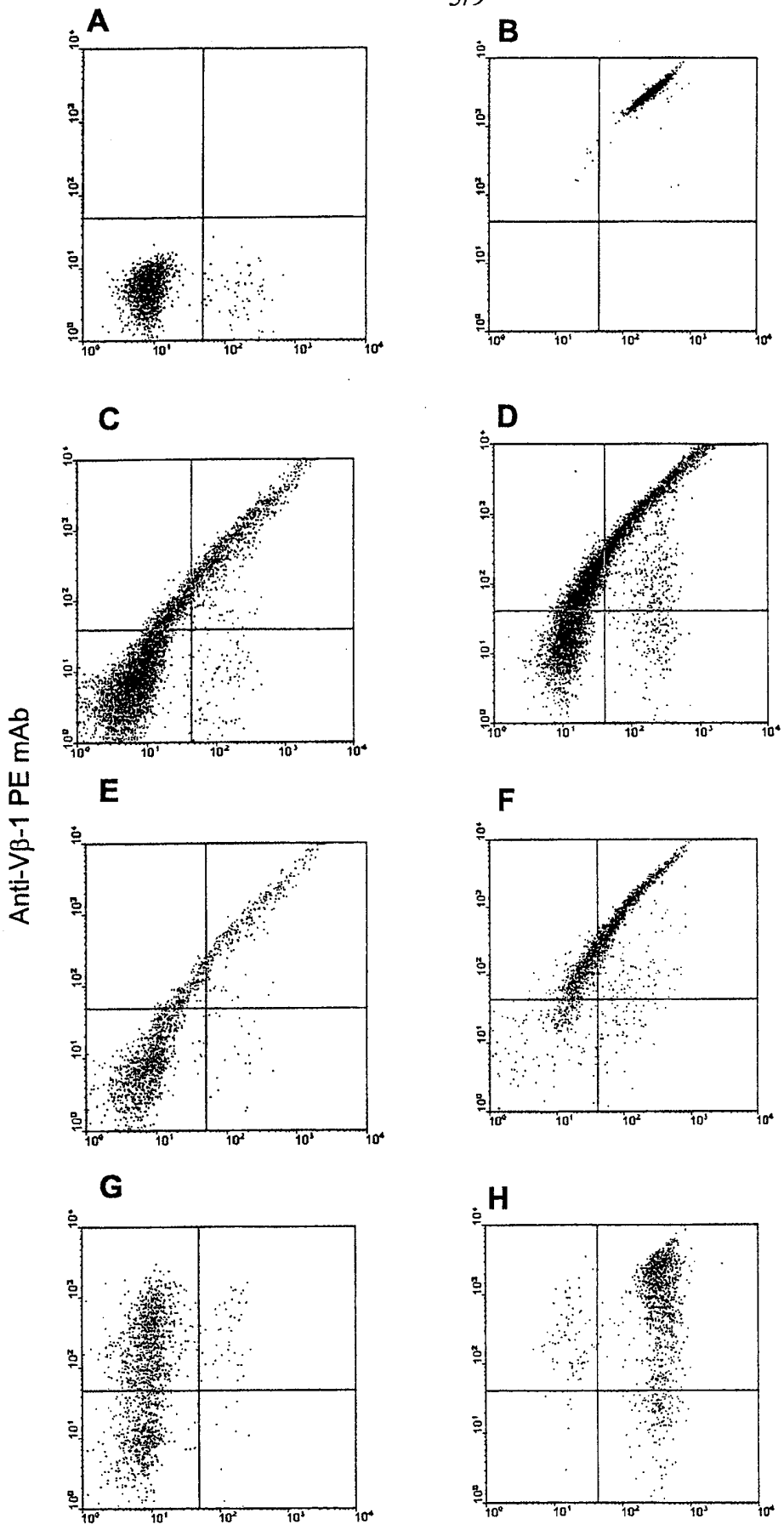


Fig. 5

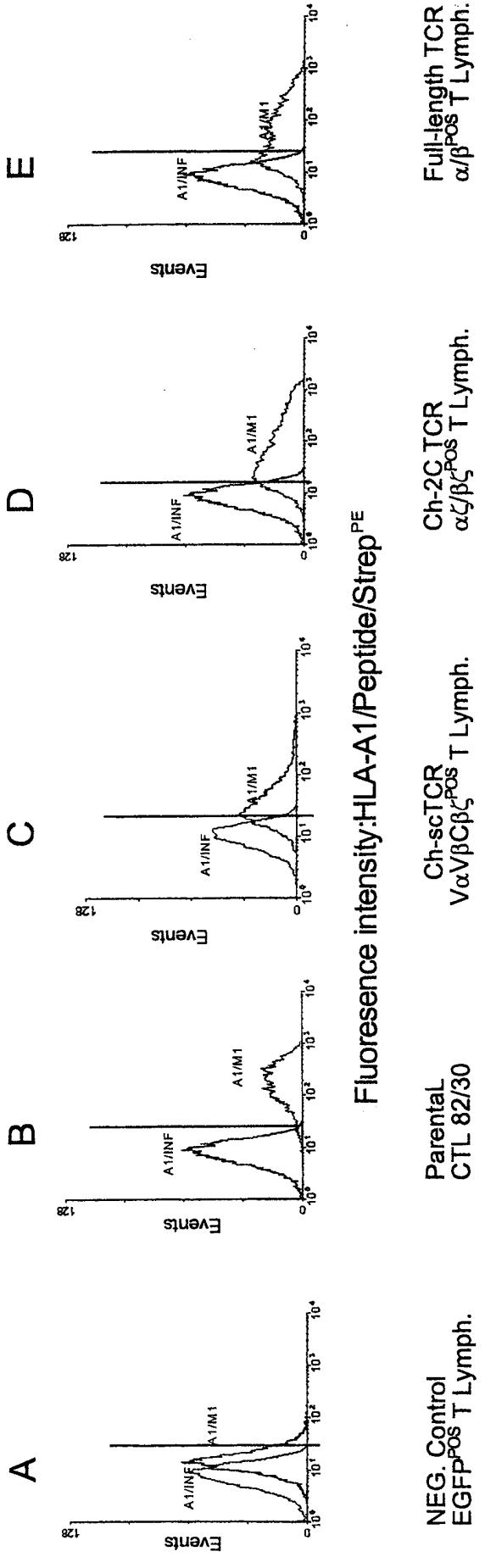

A
B
C
D
E
Events
A1/INF
A1/M1
Fluoresence intensity:HLA-A1/Peptide/Strep PE
NEG. Control
EGFP POS T Lymph.
ParentaL
CTL 82/30
Ch-scTCR
VαVβCβζ POS T Lymph.
Ch-2C TCR
αζ/βζ POS T Lymph.
Full-length TCR
α/β POS T Lymph.

Fig. 6

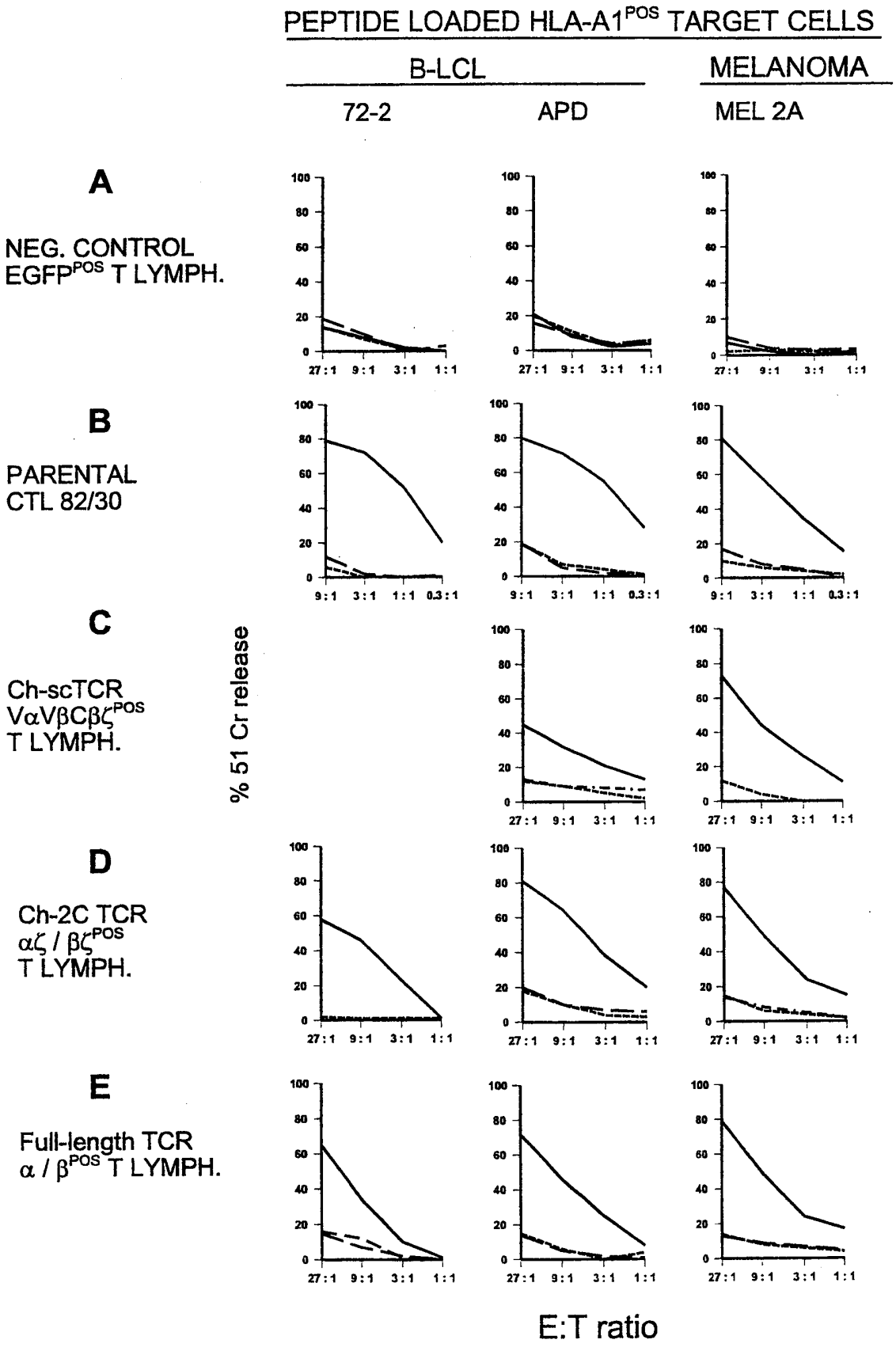

PEPTIDE LOADED HLA-A1POS TARGET CELLS
B-LCL
MELANOMA
72-2
APD
MEL 2A
A
NEG. CONTROL
EGFPPOS T LYMPH.
B
PARENTAL
CTL 82/30
C
Ch-scTCR
VαVβCβζPOS
T LYMPH.
D
Ch-2C TCR
αζ / βζPOS
T LYMPH.
E
Full-length TCR
α / βPOS T LYMPH.
% 51 Cr release
E:T ratio

Fig. 7

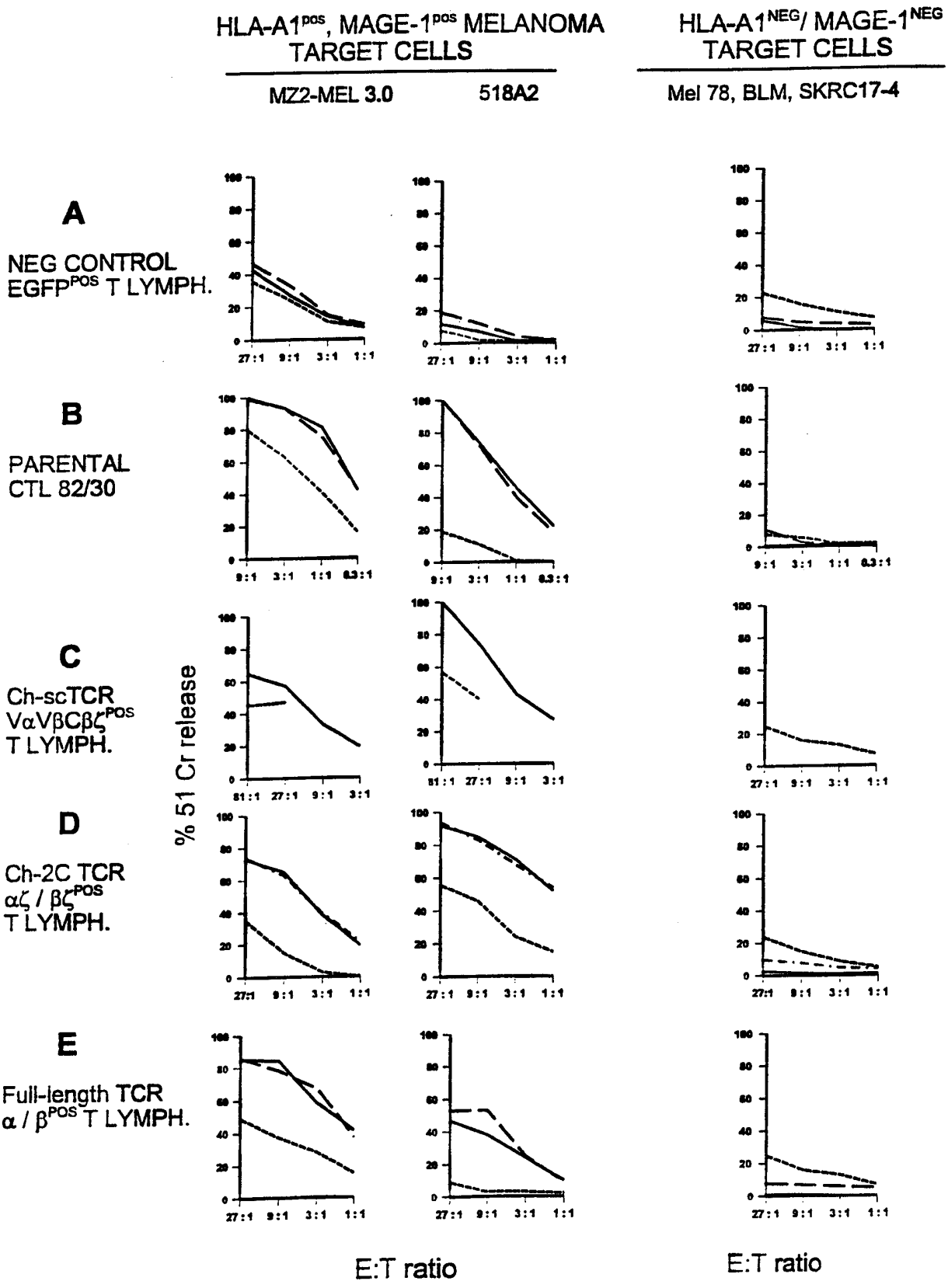

HLA-A1pos, MAGE-1pos MELANOMA TARGET CELLS
MZ2-MEL 3.0
518A2
HLA-A1NEG/ MAGE-1NEG TARGET CELLS
Mel 78, BLM, SKRC17-4
A
NEG CONTROL EGFPPOS T LYMPH.
B
PARENTAL CTL 82/30
C
Ch-scTCR VαVβCβζPOS T LYMPH.
D
Ch-2C TCR αζ / βζPOS T LYMPH.
E
Full-length TCR α / βPOS T LYMPH.
% 51 Cr release
E:T ratio
E:T ratio

Fig. 8

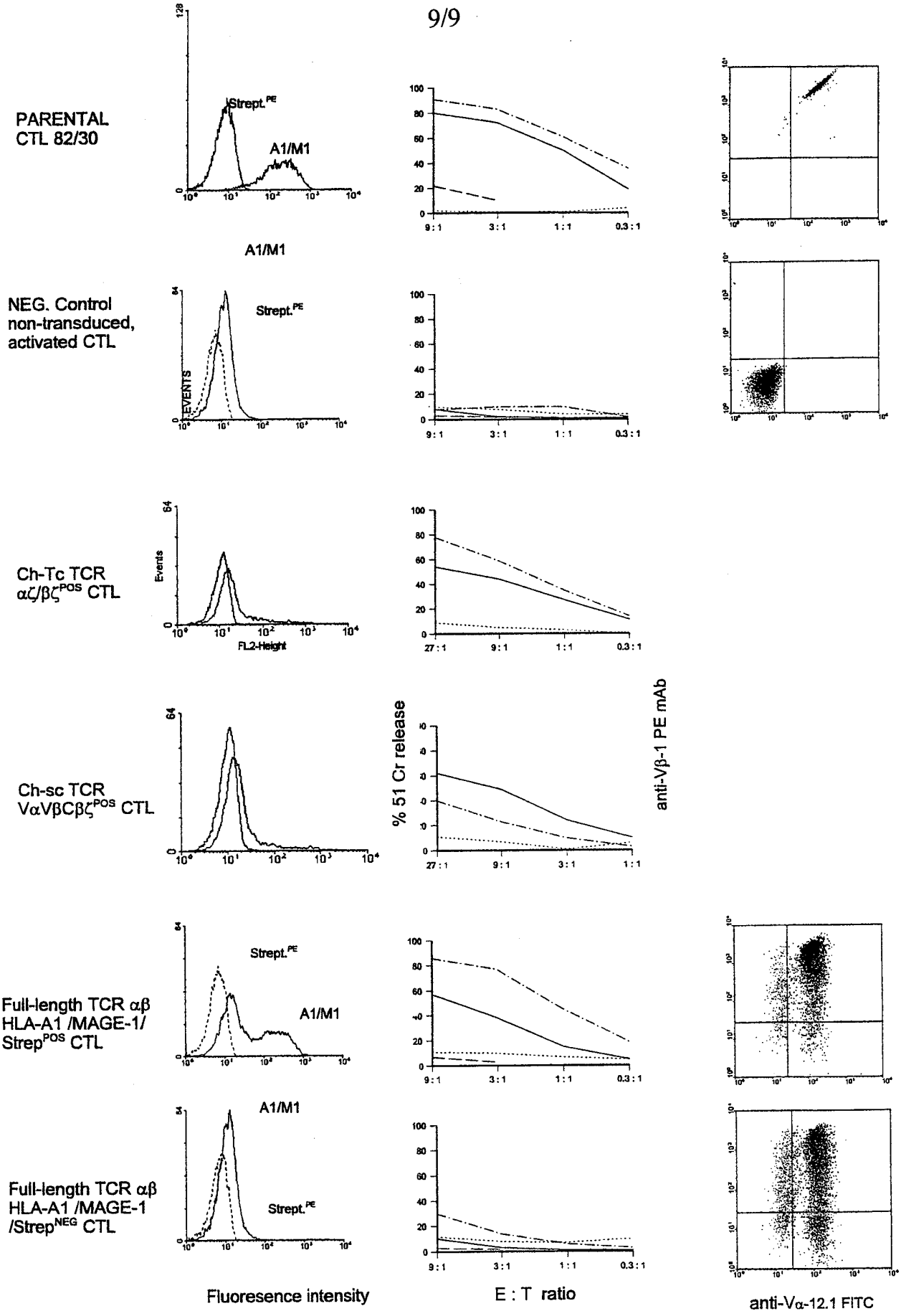

PARENTAL CTL 82/30
Strept.PE
A1/M1
A1/M1
NEG. Control non-transduced, activated CTL
Strept.PE
EVENTS
Ch-Tc TCR αζ/βζPOS CTL
Events
FL2-Height
Ch-sc TCR VαVβCβζPOS CTL
% 51 Cr release
anti-Vβ-1 PE mAb
Full-length TCR αβ HLA-A1 /MAGE-1/ StrepPOS CTL
Strept.PE
A1/M1
Full-length TCR αβ HLA-A1 /MAGE-1 /StrepNEG CTL
A1/M1
Strept.PE
9:1
3:1
1:1
0.3:1
27:1
Fluoresence intensity
E : T ratio
anti-Vα-12.1 FITC

Fig. 9

INTERNATIONAL SEARCH REPORT

Internatior `pplication No
PCT/IL 99/00622

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N5/10 C07K14/725 C07K19/00 A61K45/00

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 18105 A (HARVARD COLLEGE) 13 June 1996 (1996-06-13) page 11, line 10 -page 17, line 9	1-4,6,7, 11-15, 17, 20-22,26
Y	the whole document	1-32

X	WO 97 32603 A (SCRIPPS RESEARCH INST) 12 September 1997 (1997-09-12) page 4, line 24 -page 8, line 18; figures 1,2	1-4, 11-15, 17, 20-23,26
Y	the whole document	1-32

	-/--	

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

[X] Patent family members are listed in annex.

° Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 April 2000	26. 04. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Bilang, J

2

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	CHUNG S ET AL: "FUNCTIONAL THREE-DOMAIN SINGLE-CHAIN T-CELL RECEPTORS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 91, no. 26, 20 December 1994 (1994-12-20), pages 12654-12658, XP002039051 ISSN: 0027-8424 the whole document	1-4,6,7, 11-15, 20-22,26
Y	WILLEMSEN RA, ET AL.: "Redirection of Human CTL by Chimeric T-Cell Receptors Specific for HLA-A1-Restricted, MAGE-1-Positive Melanoma Cells" NATURAL IMMUNITY, vol. 14, November 1995 (1995-11), page 93 XP000882655 abstract	1-32
A	WO 94 29438 A (CELL GENESYS INC) 22 December 1994 (1994-12-22) cited in the application the whole document	
A	WEIJTENS ME, 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, vol. 5, no. 9, September 1998 (1998-09), pages 1195-1203, XP000882695 cited in the application the whole document	
A	ESHHAR Z ET AL: "SPECIFIC ACTIVATION AND TARGETING OF CYTOTOXIC LYMPHOCYTES THROUGH CHIMERIC SINGLE CHAINS CONSISTING OF ANTIBODY-BINDING DOMAINS AND THE GAMMA OR ZETA SUBUNITS OF THE IMMUNOGLOBULIN AND T-CELL RECEPTORS" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 90, no. 2, 15 January 1993 (1993-01-15), pages 720-724, XP002009770 ISSN: 0027-8424 the whole document	

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IL 99/00622

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Although claims 31 and 32 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internatior pplication No
PCT/IL 99/00622

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9618105 A	13-06-1996	NONE	
WO 9732603 A	12-09-1997	AU 2199797 A	22-09-1997
		CA 2246333 A	12-09-1997
		EP 0910409 A	28-04-1999
WO 9429438 A	22-12-1994	US 5834256 A	10-11-1998
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		AU 7246294 A	03-01-1995
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		EP 0710280 A	08-05-1996
		JP 8511169 T	26-11-1996
		SG 55158 A	21-12-1998
		US 5686279 A	11-11-1997
		US 5858740 A	12-01-1999