RESEARCH ARTICLE

Redirecting human CD4⁺ T lymphocytes to the MHC class I-restricted melanoma antigen MAGE-A1 by TCR $\alpha\beta$ gene transfer requires CD8 α

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Adoptive immunotherapy involving the transfer of autologous tumor or virus-reactive T lymphocytes has demonstrated its effectiveness in the eradication of cancer and virally infected cells. Clinical trials and *in vitro* studies have focused on CD8⁺ cytotoxic T-cell receptor (TCR) $\alpha\beta$ lymphocytes since these cells directly kill virally infected- and tumor cells after antigen-specific recognition via their TCR $\alpha\beta$. However, increasing evidence suggests that induction of sustained immunity against cancer and viral infections depends on the presence of tumor- or virus-specific CD4⁺ T lymphocytes, which are restricted by MHC class II. Here, we show that these MHC class II-restricted CD4⁺ T lymphocytes can efficiently be redirected to MHC class I-restricted tumor cells by retroviral introduction of an HLA-A1/MAGE-A1-specific chimeric two-chain TCR V α C α ζ /V β C β ζ (tcTCR/ ζ). However, TCR-transduced CD4⁺ T lymphocytes were only able to specifically bind to HLA-A1/MAGE-A1 complexes and respond to HLA-

A1⁺/MAGE-A1⁺ melanoma cells when the CD8 α gene was cointroduced. These CD4⁺/CD8 α ⁺/TCR^{POS} T lymphocytes produce IFN- γ , TNF α and IL-2 when specifically stimulated via the introduced TCR with immobilized HLA-A1/MAGE-A1 complexes or HLA-A1⁺/MAGE-A1⁺ melanoma cells. Furthermore, introduction of the CD8 α gene into TCR^{POS} T lymphocytes rendered these T lymphocytes cytotoxic for HLA-A1⁺/MAGE-A1⁺ melanoma cells. These results demonstrate that human CD4⁺ T lymphocytes when genetically grafted with an HLA-A1/MAGE-A1-specific TCR and CD8 α are induced to kill and produce cytokines upon specific interaction with the relevant melanoma cells. Hence, CD4⁺ T lymphocytes, in addition to CD8⁺ T lymphocytes, may be critical effector cells for adoptive immuno-gene therapy to generate a sustained tumor-specific immune response in cancer patients. Gene Therapy (2005) 12, 140–146. doi:10.1038/sj.gt.3302388 Published online 21 October 2004

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Introduction

Although both CD4⁺ and CD8⁺ T lymphocytes are involved in the immune response against cancer and viral infections, there has been a preferential attention to the antitumor responses mediated by MHC class I-restricted, CD8⁺ T lymphocytes. This focus on CD8⁺ T lymphocytes stems from experimental data showing that (i) many tumors express MHC class I but not MHC class II molecules¹ and (ii) CD8⁺ T lymphocytes are capable to directly kill tumor or virally infected cells upon recognition of MHC-presented antigenic peptides.² Moreover, (clinical) adoptive immunotherapy studies demonstrated that the transfer of viral or tumor-reactive CD8⁺ T lymphocytes can result in effective antiviral and antitumor responses, respectively.^{3–7} In addition, vaccination studies using MHC class I immuno-dominant peptides have shown that protective antitumor immunity can be generated.^{8,9} On the other hand, the important role of CD4⁺ T lymphocytes in the induction and persistence of effective immunity was demonstrated in studies using

CMV-specific CTL clones as well as CD4 knockout mice.^{10–15} After adoptive transfer of CMV-specific CD8⁺ CTL to patients, which lacked CD4⁺ CMV-specific T lymphocytes, the CD8⁺ T-cell response to CMV declined, suggesting an essential role for CD4⁺ T-helper lymphocytes in the persistence of the transferred CD8⁺ CTLs.^{10,11} The efficacy of the infused CD8⁺ CTL was improved by the coadministration of the Th1-type cytokine IL-2, supporting the notion of collaborations between CD4 and CD8 T cells.¹¹ Furthermore, mice deficient for CD4 were shown to be unable to maintain lymphocytic choriomeningitis virus (LCMV)-specific CD8⁺ CTL or clear virus following a challenge with low-dose LCMV.¹² Additionally, the induction of tumor-specific CTL against autologous MHC class II-negative effusion-associated mononuclear cell tumors was shown to be severely impaired, indicating that CD4⁺ T lymphocytes are essential for the induction of protective antitumor CD8⁺ CTL.¹⁶ Adoptive transfer of CD4⁺ tumor-specific T lymphocytes may therefore be clinically relevant for effective antitumor responses. Indeed, in mice, the adoptive transfer of activated MHC class II-restricted, tumor-specific CD4⁺ T lymphocytes has resulted in *de novo* generation of tumor-specific CD8⁺ T lymphocytes and effective antitumor responses.¹⁷ In addition, adoptive transfer of vesicular stomatitis virus-specific (VSV) CD4⁺ T lymphocytes into mice

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conferred protection against infection with a vaccinia virus expressing the VSV-G protein.¹⁸

Nevertheless, the generation of tumor-specific MHC class II-restricted CD4⁺ T lymphocytes is hampered by the lack of well-defined MHC class II-binding peptides. Powerful techniques, such as reversed immunology, used to identify MHC class I-restricted peptide epitopes¹⁹ are not yet available for the identification of MHC class II-binding peptides. Moreover, the isolation of tumor-specific MHC class II-restricted CD4⁺ T lymphocytes from individual patients is troublesome and unpredictable, and hence limits its clinical application.

We have recently demonstrated the reproducible and efficient transfer of HLA-A1-restricted, MAGE-A1-specific chimeric T-cell receptor (TCR) genes to bulk cultured primary human T lymphocytes.²⁰ Chimeric single-chain- and two-chain TCR coupled to CD3- ζ were functionally expressed on CD8⁺ T lymphocytes and have the advantage that they do not associate with endogenous TCR $\alpha\beta$ chains, when compared with full-length TCR $\alpha\beta$.^{20,21} Upon incubation with HLA-A1⁺, MAGE-A1⁺ melanoma cells, these transduced T lymphocytes were specifically activated to lyse the relevant tumor cells and to produce cytokines. In this study, we addressed whether CD4⁺ T lymphocytes can be grafted with MHC class I-restricted tumor specificity so that triggering via the MHC Class I-restricted TCR induces CD4 helper functions. We demonstrate that primary activated CD4⁺ T lymphocytes can be efficiently retargeted to HLA-A1-restricted, MAGE-A1⁺ melanoma cells by retroviral introduction of an HLA-A1/MAGE-A1-specific chimeric two-chain TCR/ ζ . However, only upon introduction of both the CD8 α gene and the tcTCR/ ζ genes, CD4⁺ T lymphocytes acquire the capacity to specifically bind to HLA-A1/MAGE-A1 complexes. In the case of CD8⁺ T lymphocytes, the CD8 molecule participates in the activation of these T lymphocytes by binding to the same peptide-MHC complex as does the TCR.^{22,23} The role of CD8 may be two-fold: (A) stabilizing the interaction between TCR and peptide-MHC complex and enhancement of the T-cell activation by increasing the $t_{1/2}$ of the TCR/CD8/peptide-MHC complex;²⁴ (B) recruitment of CD8/p56lck to the TCR/CD3/ ζ complex, which is essential for early signaling events in the T lymphocytes.^{25,26}

We demonstrated that CD4⁺/CD8 α ⁺/tcTCR/ ζ ^{POS} T lymphocytes specifically respond to immobilized HLA-A1/MAGE-A1 complexes and HLA-A1⁺/MAGE-A1⁺ melanoma cells, that is, they produce IFN- γ , TNF α and IL-2. Moreover, CD4⁺/CD8 α ⁺/tcTCR/ ζ ^{POS} T lymphocytes also specifically killed HLA-A1^{POS}, MAGE-A1^{POS} melanoma cells. Therefore, Class II-restricted human CD4⁺ T lymphocytes grafted with a class I-specific tcTCR/ ζ plus CD8 α exert immune functions that are expected to be important to provide sustained antitumor responses in patients following their adoptive transfer.

Results

Retroviral transduction of primary human T lymphocytes

Bulk populations of anti-CD3 mAb-activated primary human T lymphocytes, derived from healthy donors, were either depleted for CD8 α ⁺ with anti-CD8 α -coated

magnetic beads or used directly for retroviral transduction with the pSTITCH tcTCR V α C α ζ and V β C β ζ retroviral vectors and the pBullet CD8 α vector, essentially as described.²⁰ Following retroviral transduction, bulk cultures of T lymphocytes were expanded in media containing recombinant human IL-2. To obtain pure CD4⁺/tcTCR/ ζ ⁺, CD8 α ⁺/tcTCR/ ζ ⁺ and CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes, transduced T lymphocytes were enriched by FACS sorting after staining with the mAbs anti-V β 1^{PE}, anti-V α 12.1^{FTTC} and anti-CD8 α ^{APC}. Enriched T lymphocyte fractions were subsequently analyzed for CD4 and CD8 α and tcTCR/ ζ expression. As shown in Figure 1a, homogeneous populations of CD8 α ⁺/CD4⁺, CD8⁺ or CD4⁺ T lymphocytes were obtained. Furthermore, these enriched T lymphocyte fractions all express the chimeric tcTCR/ ζ (Figure 1b).

Expression of CD8 α on CD4⁺/tcTCR/ ζ ⁺ T lymphocytes is required for binding to MAGE-A1/HLA-A1 tetramers

Specific binding to MAGE-A1/HLA-A1 tetramers by flow-sorted CD4⁺/tcTCR/ ζ ⁺, CD8 α ⁺/tcTCR/ ζ ⁺ and CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes was determined by flow-cytometry analysis. Only those CD4⁺ T lymphocytes that express both CD8 α and tcTCR/ ζ retroviral constructs specifically bound MAGE-A1/HLA-A1 tetramers (M1/A1). CD4⁺ T lymphocytes that lacked CD8 α did not bind tetramers (Figure 2a). CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes and the CTL clone MZ2-82/30 from which the TCR α and β genes were cloned¹⁹ served as positive controls and specifically bound the M1/A1 tetramers (Figure 2b). The simultaneous addition of anti-CD8 α mAb and M1/A1 tetramers to the tcTCR/ ζ ⁺ T lymphocytes resulted in a significant reduction of binding to the M1/A1 tetramer, demonstrating that CD8 α is required for the TCR/MHC interaction (Figure 2c). Specificity of binding was confirmed by the lack of binding by transduced T lymphocytes to tetramers that contained an irrelevant Influenza virus peptide derived from Influenza virus A nucleoprotein (Flu/A1) (data not shown).

CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes specifically respond to native MAGE-A1⁺, HLA-A1⁺ melanoma cells by TNF α , IFN- γ and IL-2 production

We then determined whether native MAGE-A1⁺, HLA-A1⁺ melanoma cells could specifically activate the CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes to produce TNF α , IFN- γ and IL-2. Therefore, CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes were incubated with (i) the MAGE-A1⁺, HLA-A1⁺ melanoma cell line MZ2-Mel3.0 (MEL 3.0), (ii) the MAGE-A1⁻, HLA-A1⁻ renal cell carcinoma cell line SKRC17 cl 4 (SKR), (iii) the HLA-A1⁻ melanoma cell lines Mel 78 and BLM, (iv) the HLA-A1⁺, MAGE-A1⁻ melanoma cell line MZ2-MEL2.2 (MEL 2.2) and (v) the HLA-A1⁺, MAGE-A1⁻ melanoma cell line MZ2-MEL2.2 loaded with MAGE-A1 peptide (10 μ g/ml final) and the EBV-transformed B cell line APD. As shown in Figure 3, only MAGE-A1⁺/HLA-A1⁺ melanoma cells specifically activated the CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes to produce TNF α , IFN- γ and IL-2. None of these cytokines were produced when CD4⁺/CD8 α ⁺/tcTCR/ ζ ⁺ T lymphocytes were incubated with MAGE-A1^{NEG} or HLA-A1^{NEG} target cell lines. Furthermore, no TNF α , IFN- γ and IL-2 was produced when CD4⁺/tcTCR/ ζ ⁺ T lymphocytes

Figure 1 Expression of (a) CD4 and CD8 α and (b) TCR V α 12.1 and V β 1 on primary human T lymphocytes transduced with the chimeric tcTCR $\alpha\zeta/\beta\zeta$ genes and the CD8 α gene. Homogeneous populations of CD4⁺/tcTCR/ζ⁺, CD4⁺/CD8⁺/tcTCR/ζ⁺ and CD8⁺/tcTCR/ζ⁺ T lymphocytes were obtained by FACS-sorting using mAbs binding to Vβ1, Vα12.1 and CD8 α . Dot plots show viable cells selected on FSC and SSC. Transduced T lymphocytes were stained and sorted as described in Materials and methods.

were incubated with the MAGE-A1⁺/HLA-A1⁺ melanoma cells (data not shown).

Lysis of 'native' MAGE-A1⁺/HLA-A1⁺ melanoma cells as well as MAGE-A1 peptide pulsed melanoma cells by CD4⁺ T lymphocytes expressing chimeric tcTCR/ζ depends on CD8 α

To determine whether CD4⁺ T lymphocytes engrafted with chimeric tcTCR/ζ are capable to kill MAGE-A1⁺/HLA-A1⁺ target cells, we incubated CD4⁺/tcTCR/ζ⁺ as well as CD4⁺/CD8 α ⁺/tcTCR/ζ⁺ T lymphocytes with ⁵¹Cr-labeled MAGE-A1⁺/HLA-A1⁺ melanoma cells and MAGE-A1 peptide-pulsed HLA-A1⁺ melanoma cells. MAGE-A1 peptide-pulsed melanoma cells and more importantly, 'native' MAGE-A1⁺/HLA-A1⁺ melanoma cells were efficiently lysed by CD4⁺/CD8 α ⁺/tcTCR/ζ⁺ T lymphocytes (Figure 4a). Melanoma cells that lack the MAGE-A1 peptide were not lysed by CD4⁺/CD8 α ⁺/tcTCR/ζ⁺ T lymphocytes. In fact, lysis of MAGE-A1 peptide-pulsed melanoma cells as well as 'native' MAGE-A1⁺/HLA-A1⁺ melanoma cells depends on the coexpression of CD8 α . CD4⁺/tcTCR/ζ⁺ T lymphocytes were unable to kill any HLA-A1⁺/MAGE-A1⁺ target cells. In addition, lysis of 'native' MAGE-A1⁺/HLA-A1⁺

melanoma cells by CD4⁺/CD8 α ⁺/tcTCR/ζ⁺ T lymphocytes as well as the parental CTL clone MZ2-82/30 is completely blocked after addition of anti-CD8 α mAb (Figure 4b).

Discussion

In this study, we explored the possibility to graft CD4⁺ T lymphocytes with an MHC class I-restricted tumor specificity by retroviral transfer of TCR genes. CD4⁺ T lymphocytes can be functionally endowed with TCR $\alpha\beta$ specific for MAGE-A1 presented by HLA-A1 molecules on melanoma cells.

These CD4⁺/tcTCR/ζ⁺ T lymphocytes were induced to produce cytokines such as TNF- α , IFN- γ and IL-2. In addition, CD4⁺/tcTCR/ζ⁺ lymphocytes exert immune-specific lysis of MAGE-A1⁺/HLA-A1⁺ melanoma cells. Importantly, we show that for HLA-A1/MAGE-A1-specific TCR binding to HLA-A1/MAGE-A1 tetramers as well as antigen-specific cytokine production and cytotoxicity CD4⁺, tcTCR/ζ⁺ lymphocytes require the coexpression of CD8 α on their cell surface.

Attempts to generate tumor-reactive human CD4⁺ T lymphocytes via nongenetic means have been ham-

Figure 2 CD4⁺ T lymphocytes expressing t α TCR/ ζ require coexpression of CD8 α to bind HLA-A1/MAGE-A1 tetramers. (a) MAGE-A1/HLA-A1 (M1/A1) tetramer staining of CD4⁺/t α TCR/ ζ ⁺ CD4⁺/CD8⁺/t α TCR/ ζ ⁺ T lymphocytes. (b) M1/A1 tetramer staining of CD8⁺/t α TCR/ ζ ⁺ T lymphocytes and the parental CTL clone MZ2-82/30. (c) Addition of anti-CD8 α (10 μ g/ml final) to CD4⁺/CD8⁺/t α TCR/ ζ ⁺ T lymphocytes and the parental CTL MZ2-82/30 blocks M1/A1 tetramer binding. T lymphocytes were stained and sorted as described in Materials and methods.

pered by the difficulty to identify MHC class II-restricted tumor epitopes that can be used for their *in vitro* stimulation. Reproducible technologies for the identification of MHC class I-presented tumor antigens have been developed,¹⁹ but these are lacking for class II-presented tumor antigens.²⁷ Owing to the fundamental differences in peptide processing and loading of the MHC class II molecules, when compared to MHC class I molecules, identification of class II-presented tumor peptides awaits a technical breakthrough.^{27,28} Despite technical difficulties, the availability of CD4⁺ T lymphocytes is of importance because CD4⁺ T lymphocytes play critical roles in the generation of antigen-specific T-cell res-

ponses and in the induction of memory, providing sustained immunity.^{13–18}

Recently, we and others successfully grafted primary human T lymphocytes with MHC class I-restricted specificity through introduction of full-length TCR $\alpha\beta$, chimeric single-chain and two-chain TCR genes. Introduction of these (chimeric) TCR conferred the T lymphocytes with a new MHC-restricted tumor specificity.^{20,29–31}

The physiological interaction between TCR $\alpha\beta$ on T lymphocytes and MHC class I molecules on target cells may depend on the simultaneous interaction of CD8 α and/or CD8 β on T lymphocytes with the MHC class I molecules on target cells.^{32–34} In fact, addition of anti-

Figure 3 HLA-A1-restricted, MAGE-A1-specific cytokine production by CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocytes. To test HLA-A1/MAGE-A1-specific production of cytokines, CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocytes were incubated for 17 h with a panel of cell lines positive or negative for HLA-A1 and MAGE-A1 (MEL 3.0 A1⁺/M1⁺, BLM A1⁻/M1⁻, MEL 78 A1⁻/M1⁺, SKRC 17 cl4 A1⁻/M1⁻, MEL-2.2 A1⁺/M1⁻, MEL-2.2 in the presence of M1 peptide (10 μ g/ml final) and APD A1⁺/M1⁻). IFN- γ , TNF- α and IL-2 production were analyzed by ELISA as described in Materials and methods. Results from one representative experiment (out of three) are shown.

CD8 mAbs generally inhibits T-cell-mediated immune functions^{33,35} or specific binding of soluble MHC class I tetramers.³⁶ We addressed the question whether CD4⁺/tcrTCR/ ζ ⁺ T lymphocytes also require CD8 α to bind tetramers and become activated by relevant target cells. After CD8 α and tcrTCR/ ζ transduction, CD4⁺/tcrTCR/ ζ ⁺, CD8 α ⁺/tcrTCR/ ζ ⁺ and CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocyte populations were sorted and expanded for analysis. Only the CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocytes, but not CD8 α ⁻/CD4⁺/tcrTCR/ ζ ⁺ T lymphocytes, were able to bind tetramers and exerted tcrTCR/ ζ -mediated immune functions, that is, cytokine production and lysis of tumor cells. The cytokines IL-2, IFN- γ and TNF α were produced only after specific stimulation with the relevant MAGE-A1/HLA-A1-expressing target cells. We conclude that triggering of the HLA-A1/MAGE-A1-specific immune response required the coexpression of

Figure 4 Lysis of native HLA-A1⁺, MAGE-A1⁺ and MAGE-A1 peptide loaded melanoma cells by CD4⁺/tcrTCR/ ζ ⁺ T lymphocytes depends on coexpression of CD8 α . (a) The HLA-A1, MAGE-A1-specific cytolytic capacity of CD4⁺/tcrTCR/ ζ ⁺ and CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocytes was analyzed in 4 h ⁵¹Cr-release assays, using HLA-A1⁺/MAGE-A1⁺ MZ2-MEL3.0 melanoma cells (MEL 3.0) and MZ2-MEL 2.2 melanoma cells in the presence of MAGE-A1 peptide (10 μ g/ml final, MEL 2.2). (b) Addition of anti-CD8 α mAb blocks HLA-A1/MAGE-A1-specific cytotoxicity of CD4⁺/CD8 α ⁺/tcrTCR/ ζ ⁺ T lymphocytes and the parental CTL MZ2-82/30. Anti-CD8 α mAb (10 μ g/ml final) was added to the T lymphocytes in 4 h ⁵¹Cr-release assays, using HLA-A1⁺/MAGE-A1⁺ MEL3.0 melanoma cells as targets. Results from one representative experiment (out of three) are shown. Assays were performed as described in Materials and methods.

CD8 α and the tcrTCR/ ζ . Indeed, the tcrTCR/ ζ -mediated target cell lysis and binding of tetramers was inhibited by the addition of anti-CD8 mAb (Figures 4b and 2c, respectively).

CD4⁺ primary human T lymphocytes grafted with MHC-antigen-independent, antibody-based scFv/ ζ or scFv/ γ receptors were also shown to be responsive to their relevant target antigen-expressing tumor cells.^{37,38} These scFv-receptor-expressing T cells also mediated cytotoxicity of antigen-positive target cells and produced TNF α and IFN γ . However, IL-2 secretion was only observed when the scFv/ ζ -receptor expressing cells were stimulated through their endogenous TCR, and not when stimulated through the antibody-based receptor.³⁷ This difference in capacity to produce IL-2 between tcrTCR/ ζ -expressing cells and scFv/ ζ -expressing cells may reflect differences in the capacity of these receptors to recruit multiple src kinase signaling molecules, phosphorylation of the receptors and eventually amplification of the receptor-mediated signals.

In mice, adoptive transfer models of viral infections such as influenza and VSV have demonstrated that the

transfer of CD4 T-cell clones effectively protected mice from influenza or VSV-induced infections.¹⁸ The CD4⁺/CD8 α ⁺/tTCR/ ζ ⁺ T lymphocytes that exert the described immune functions may also exert help to generate CD8⁺ CTL *in vivo* as well as memory responses.³⁹ In a clinical study, it was demonstrated that long-term persistence of adoptive transferred cytomegalovirus or human immunodeficiency virus-specific CD8⁺ CTL probably requires adequate CD4 T-helper functions.¹¹ Taken these clinical data and data obtained from adoptive transfer studies in CD4 knockout mice together, the adoptive transfer of both CD8 α ⁺/tTCR/ ζ ⁺ T lymphocytes and CD4⁺/CD8 α ⁺/tTCR/ ζ ⁺ T lymphocytes may prolong *in vivo* survival of the transferred CD8⁺ CTL, which might overcome the requirement of repetitive infusions of high doses of CD8⁺ T cells. Furthermore, infusions of CD4⁺ T cells may help the *de novo* generation of patients CD8⁺ CTL with tumor specificity. Finally, in addition to providing help to CD8⁺ effector T cells, CD4⁺ cells equipped with MHC class I specificity may enhance therapeutic efficacy via direct or indirect effector functions.

Materials and methods

Cells and antibodies

T lymphocytes derived from healthy donors were isolated and expanded as described elsewhere.⁴⁰ Target cell lines used in this study are (i) the MAGE-A1⁺/HLA-A1⁺ melanoma cell line MZ2-MEL.3.0 (MEL 3.0); (ii) the MAGE-A1⁻/HLA-A1⁺ melanoma cell line MZ2-MEL 2.2 (MEL 2.2) (kindly provided by T Boon and P Coulie, Brussels, Belgium;¹⁹ (iii) the HLA-A1⁻ melanoma cell line MEL 78; (vi) the HLA-A1⁻ melanoma cell line BLM (kindly provided by G Adema, Nijmegen, the Netherlands); (vii) the HLA-A1⁻ renal carcinoma cell line SKR17-4 (SKR) (kindly provided by E Oosterwijk, Nijmegen, the Netherlands) and (viii) the HLA-A1⁺ EBV-transformed B-cell line APD. The human embryonic kidney cell line 293T⁴¹ and phoenix-amph⁴² were used for production of pStitch V α C α ζ /V β C β ζ and pBullet CD8 α retroviral vectors. The mAbs used in this study were anti-CD4^{FITC} anti-CD8 α ^{PE/APC} (Becton Dickinson Biosciences, San Jose, CA, USA), anti-CD8 α for blocking experiments (CLB, Amsterdam, the Netherlands); the TCR V α and V β family-specific mAbs anti-V α 12.1^{FITC} (T Cell Diagnostics, USA) and anti-V β 1^{PE} (Coulter-Immunotech, Marseille, France). HLA-A1/MAGE-A1 tetramers were kindly provided by P van der Bruggen (Brussels, Belgium).

Construction of retroviral vectors and transduction of primary human T lymphocytes

The chimeric TCR V α C α ζ and V β C β ζ genes were derived from the CTL clone MZ2-82/30 as described previously.²⁰ The CD8 α gene was amplified with primers that allowed introduction into the retroviral vector pBullet, from the pLCD8 α SH vector (a kind gift from Jos Molenhorst, Leiden, the Netherlands). Chimeric TCR genes and the CD8 α gene were introduced into populations of anti-CD3 mAb-activated primary human T lymphocytes, which were either depleted with anti-CD α mAb-coated magnetic beads or used directly, essentially as described.²⁰ After two rounds of super-

natant transduction, the transduced T lymphocytes were expanded in culture medium, supplemented with r-IL2 (360 IU/ml). All experiments were performed with primary human T lymphocytes obtained from at least five healthy donors.

Flow-cytometry analysis and sorting of retrovirally transduced human T lymphocytes

Viable human T lymphocytes (5×10^5) were stained with TCR V α and V β family-type-specific anti-V α 12.1^{FITC} (2 μ g/ml) and anti-V β 1^{PE} (2 μ g/ml) mAb in a final volume of 50 μ l. Transduced T lymphocytes (5×10^5) were stained with MAGE-A1/HLA-A1 tetramers (10 nM final, 30 min on ice). T lymphocyte viability was assessed by trypan blue staining: only T lymphocyte populations >95% viable were used for flow-cytometry analysis. The dot plots represent 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). FACS sorting was performed on a FACS-Vantage instrument (Becton Dickinson Biosciences) using saturating concentrations of V α 12.1^{FITC}, V β 1^{PE} and CD8 α ^{APC} mAb. Pure populations of CD4⁺/tTCR/ ζ ⁺, CD8 α ⁺/tTCR/ ζ ⁺ and CD4⁺/CD8 α ⁺/tTCR/ ζ ⁺ T lymphocytes were obtained by selecting for T lymphocytes that were V α 12.1⁺, V β 1⁺ and CD8 α ⁺ or ⁻, starting with CD8 α -depleted or nondepleted lymphocyte populations. FACS-sorted lymphocytes were expanded prior to their use in functional assays as described.²⁰

TNF- α , IFN- γ and IL-2 production

To quantify TNF- α , IFN- γ and IL-2 production by the transduced flow-sorted human T lymphocytes, 6×10^4 transduced T lymphocytes were cultured for 24 h either in the presence or absence of 2×10^4 adherent tumor cells in RPMI-1640 medium supplemented with 360 IU/ml rIL-2, and where indicated, with 10 μ g/ml MAGE-A1 peptide. At the end of culture, supernatant was harvested and the levels of TNF- α , IFN- γ and IL-2 (CLB, Amsterdam, the Netherlands) were measured by standard ELISA according to the manufacturer's instructions.

Cytotoxicity assays

Cytolytic activity of transduced human T lymphocytes was measured in ⁵¹Cr-release assays as described previously.²⁰ Peptide loading of target cells was performed by adding MAGE-A1 nonapeptide (EADPTGHSY, Leiden University Medical Center, the Netherlands) or irrelevant HLA-A1-binding Influenza peptide (CTELKLSY, Leiden University Medical Center, the Netherlands) at the indicated concentrations to the target cells 5–15 min prior to incubation with effector T lymphocytes. The mAb anti-CD8 α (10 μ g/ml final) was added to the melanoma target cells 5 min before incubation with the T lymphocytes. Effector and target cells were cultured at the indicated E:T ratios. The incubation period of effector and target cells is indicated in the figures. Percentage specific ⁵¹Cr release was calculated as follows: ((test counts – spontaneous counts) / (maximum counts – spontaneous counts)) $\times$ 100%.

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