Peptide Fine Specificity of Anti-Glycoprotein 100 CTL Is Preserved Following Transfer of Engineered TCR$\alpha\beta$ Genes Into Primary Human T Lymphocytes[1]

Niels Schaft,* Ralph A. Willemsen,* Jolanda de Vries,† Birgit Lankiewicz,* Bram W. L. Essers,† Jan-Willem Gratama,* Carl G. Figdor,† Reinier L. H. Bolhuis,* Reno Debets,[2]* and Gosse J. Adema†

TCR with known antitumor reactivity can be genetically introduced into primary human T lymphocytes and provide promising tools for immunogene therapy of tumors. We molecularly characterized two distinct TCRs specific for the same HLA-A2-restricted peptide derived from the melanocyte differentiation Ag gp100, yet exhibiting different stringencies in peptide requirements. The existence of these two distinct gp100-specific TCRs allowed us to study the preservation of peptide fine specificity of native TCR$\alpha\beta$ when engineered for TCR gene transfer into human T lymphocytes. Retroviral transduction of primary human T lymphocytes with either one of the two sets of TCR$\alpha\beta$ constructs enabled T lymphocytes to specifically kill and produce TNF-α when triggered by native gp100^{pos}/HLA-A2^{pos} tumor target cells as well as gp100 peptide-loaded HLA-A2^{pos} tumor cells. Peptide titration studies revealed that the cytolytic efficiencies of the T lymphocyte transductants were in the same range as those of the parental CTL clones. Moreover, primary human T lymphocytes expressing either one of the two engineered gp100-specific TCRs show cytolytic activities in response to a large panel of peptide mutants that are identical with those of the parental CTL. The finding that two gp100-specific TCR, derived from two different CTL, can be functionally introduced into primary human T lymphocytes without loss of the Ag reactivity and peptide fine specificity, holds great promise for the application of TCR gene transfer in cancer immunotherapy. *The Journal of Immunology,* **2003, 170: 2186–2194.**

Cytotoxic T lymphocytes specific for tumor-associated Ags have the capacity to mediate efficient antitumor immune responses in vivo. The availability of tumor-specific CTLs enabled the molecular identification and characterization of a large panel of class I MHC-binding peptides derived from tumor and viral Ags (for reviews, see Refs. 1 and 2). Of the melanoma differentiation Ag gp100, multiple peptides have been identified that are recognized by CTL isolated either from peripheral blood or tumor lesions of melanoma patients (3–6). Recently, we have demonstrated that it is possible to generate specific CTL directed against gp100 using peptide-pulsed APC and PBL of healthy individuals (5). In these studies, we made use of dendritic cells (DCs)[3] as the APC. DCs are key regulators in immune responses, capable of priming naive T cells, which, at least in part, is due to their high expression of MHC class I and II molecules as well as costimulatory molecules (7–9). Currently, DC-based vaccines are used to induce antitumor immunity in man.

Alternatively, adoptive transfer of Ag-specific, MHC-restricted CTL may be successful in the eradication of tumors in patients with metastatic melanoma and in e.g., allogeneic bone marrow transplant recipients with EBV-positive tumors (10–13). Unfortunately, patients do not always mount a strong and effective in vivo cytotoxic T cell response to their tumors. In fact, isolation of tumor-specific T lymphocytes has only been possible in a fraction of patients, most likely due to the fact that, at least in an immunocompetent setting, the peripheral T cell repertoire is devoid of high-avidity tumor-specific CTL due to thymic selection (14). In addition, these cells only have a limited lifespan, and expansion of such T lymphocytes to therapeutic doses is often not feasible (15, 16). To overcome these problems, T lymphocytes can be harnessed with antitumor/virus specificities via genetic means. Genes encoding for TCR α- and β-chain can be cloned from the tumor-specific CTL clones, and transfer of these TCR$\alpha\beta$ genes into primary human T lymphocytes reprograms T lymphocytes with a specificity against a tumor Ag. In several in vitro studies, primary human T lymphocytes have been retargeted with a tumor or virus specificity by transfer of TCR$\alpha\beta$ genes, and were shown to respond specifically toward target cells expressing both the corresponding Ag and the correct HLA molecule (17–22). Recently, it was also shown that mouse T cells that were redirected by TCR gene transfer efficiently promoted the rejection of Ag-expressing tumors in vivo (23).

To date, very little is known about the preservation of both the cytotoxic potency and peptide fine specificity of CTL following TCR gene transfer, which is crucial to the successful use of TCR genes for immunogene therapy. In this study, we describe the characterization of the TCRs of two anti-gp100/HLA-A2 CTL clones: one obtained from PBL of a healthy donor via stimulation with peptide-pulsed DC (5), and the other obtained from PBL of a melanoma

*Clinical and Tumor Immunology, Department of Medical Oncology, Erasmus Medical Center-Daniel den Hoed, Rotterdam, The Netherlands; and †Department of Tumor Immunology, University Medical Center, Nijmegen, The Netherlands

Received for publication March 1, 2002. Accepted for publication December 2, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This work was partly supported by the Faculty of Medical Sciences (Nijmegen) and the Dutch Cancer Society.

[2] Address correspondence and reprint requests to Dr. Reno Debets, Clinical and Tumor Immunology, Department of Medical Oncology, Erasmus Medical Center-Daniel den Hoed, Groene Hilledijk 301, 3075 EA Rotterdam, The Netherlands. E-mail address: Debets@immh.azr.nl

[3] Abbreviations used in this paper: DC, dendritic cell; B-LCL, B lymphoblast cell line; CDR, complementarity-determining region; fl, full length; mIg, mouse γ globulin; wt, wild type; FL, fluorescein.

0022-1767/03/$02.00

patient via stimulation with autologous melanoma cells (24). These CTL clones showed identical cytotoxic responses against gp100^{pos}/HLA-A2^{pos} target cells, but clearly displayed different fine specificities for gp100 amino acid substitution mutants. The existence of two gene sets each encoding for TCR$\alpha\beta$ molecules specific for gp100 peptide but with distinct gp100 peptide fine specificities enabled us to analyze the preservation of both the cytotoxic potency and peptide fine specificity of the physiological TCRs when engineered for TCR gene transfer into human T lymphocytes. Peptide-loaded HLA-A2^{pos} and native gp100^{pos}/HLA-A2^{pos} tumor target cells triggered lysis and TNF-α production by TCR-transduced T lymphocytes. The cytolytic efficiencies of the TCR-transduced T lymphocytes were in the same range as those of the parental CTL clones. Most importantly, the gp100 peptide fine specificities of the transduced TCRs were identical with those of the parental CTLs. Taken together, this report shows for the first time that human TCR genes can be functionally transferred to human T lymphocytes without loss of cytolytic efficiency and peptide fine specificity.

Materials and Methods

Cells and cell lines

PBLs from healthy donors were isolated by centrifugation through Ficoll-Isopaque (density = 1.077 g/cm^3; Amersham Pharmacia Biotech, Uppsala, Sweden). Transduced primary human T lymphocytes were cultured in RPMI 1640 medium supplemented with 25 mM HEPES, 200 mM L-glutamine, 10% human serum, and 360 IU/ml human rIL-2 (Proleukin; Chiron, Amsterdam, The Netherlands), and stimulated every 2 wk with a mixture of irradiated allogeneic feeder cells, as described elsewhere (25). The melanoma cell lines BLM (HLA-A2^{pos}), BLM transfected with human gp100-encoding cDNA (BLMgp100), and MEL624 (gp100^{pos}/HLA-A2^{pos}) were cultured as described previously (26, 27). The human amphotropic packaging cell line Phoenix, the melanoma cell lines FM3 (gp100^{pos}/HLA-A2^{pos}; a kind gift from J. Zeuthen, Danish Cancer Society, Copenhagen, Denmark) and LB265-MEL, as well as the TAP-deficient T $\times$ B cell hybrid T2 cells (HLA-A2^{pos}) were grown in DMEM (Life Technologies, Paisley, U.K.) supplemented with 10% bovine calf serum (HyClone, Logan, UT). The B lymphoblast cell line (B-LCL) BSM (HLA-A2^{pos}) and K562, a chronic myelogenic leukemic cell line, were cultured in RPMI 1640 medium supplemented with 200 mM L-glutamine and 10% bovine calf serum. The B cell lines LG2-EBV and JY-EBV were cultured in IMDM as described previously (28).

Contamination of cell lines and cells with mycoplasm species was excluded by frequent PCR testing of cellular DNA with mycoplasm specific primers (Gen-Probe, San Diego, CA). HLA-A2 expression of melanoma cell lines was checked by flow cytometry and expression of gp100 was checked by staining cytospin preparations with anti-gp100 polyclonal Ab (HMB45).

Peptides and peptide binding assays

Peptides used in this study were: an irrelevant HLA-A2-binding EBV-peptide (GLCTLVAML), gp100$_{280-288}$ wild-type (wt) peptide YLEPGPVTA, and the gp100 peptide mutants (A1 to A8, and G9, with the A or G referring to an alanine or glycine residue, respectively, and the number corresponding to the position of the amino acid in the gp100 wt peptide). Peptides were synthesized with a free C terminus either by F-moc peptide chemistry using an ABIMED Multiple Synthesizer (Abimed Analyes-Technik, Langenfeld, Germany) or by T-boc chemistry on a Biosearch SAM2 peptide synthesizer (Biosearch Technologies, Novato, CA). All peptide preparations were >90% pure as analyzed by analytical HPLC. Peptides were dissolved in 100% DMSO and stored at −20°C. The HLA-A2 stabilization assay on T2 cells has been described previously (29). Briefly, peptides at various dilutions were incubated with 10^5 T2 cells for 14 h at 37°C/5% CO_2 in serum-free medium in the presence of 3 μg/ml human β_2-microglobulin (Sigma-Aldrich, St. Louis, MO) in a total volume of 100 μl. Peptide-mediated stabilization of HLA-A2 molecules at the cell surface of T2 cells was analyzed by flow cytometry using the anti-HLA-A2 mAb BB7.2, and expressed as a fluorescence index: (experimental mean fluorescence/background mean fluorescence) − 1. The background mean fluorescence values were obtained by incubating T2 cells with an HLA-A2 nonbinding peptide (WMAFKERKV) at a similar concentration as the experimental peptide. A second, competition-based HLA-A2 peptide binding assay using JY-EBV cells was performed as described previously (30). In short, HLA molecules on JY-EBV cells were stripped via a mild acid treatment. Stripped cells were subsequently incubated with various concentrations of peptides for 24 h at 4°C in the presence of 150 nM of a fluorescein (FL)-labeled reference peptide (FLPSDC(-FL)FPSV) and 1.5 μg/ml human β_2-microglobulin, and analyzed by flow cytometry. The binding capacity of a peptide is expressed as the concentration required to inhibit 50% of the binding of the FL-labeled reference peptide (IC_{50}).

Generation of CTL

CTL-MPD, specific for the HLA-A2-presented gp100$_{280-288}$ peptide, was cloned via limiting dilution from a bulk anti-gp100 CTL culture that was induced in vitro using gp100 wt peptide-pulsed DC as described previously (5). After several weeks of restimulation, stable clones were obtained. CTL-MPD was expanded weekly: CTL were mixed with LB265-MEL, LG2-EBV, and JY-EBV cells, the latter two pulsed with the gp100$_{280-288}$ wt peptide, in IMDM supplemented with 3% human serum, 120 IU/ml human rIL-2, and 5 ng/ml human rIL-7 (Genzyme, Cambridge, MA). CTL-296, also specific for the HLA-A2-presented gp100$_{280-288}$ peptide, was generated from PBL of a melanoma patient (24). The CTL-296 was maintained via coculture of this CTL with gp100 wt peptide-pulsed T2 cells, LG2-EBV and JY-EBV cells in IMDM containing 10% human serum, 200 mM L-glutamine, and 50 IU human rIL-2. The CTL cultures were restimulated weekly.

Analysis of TCR Vα/Vβ gene usage of CTL-MPD and CTL-296

RNA was isolated from 10^6 CTL and used for reverse transcriptase reactions performed with Superscript (Life Technologies) according to the manufacturer's instructions. The TCR α-chain variable regions were amplified using a set of sense primers specific for the TCRAV1 to the TCRAV29 segment (kindly provided by Dr. T. Logtenberg, Crucell bv, Leiden, The Netherlands, and Dr. P. van der Elsen, Leiden University Medical Center, Leiden, The Netherlands) in combination with a TCRAC antisense consensus primer. Nested PCR was performed on TCRVA products before gel electrophoresis. The TCR β-chain variable regions were amplified using a combination of degenerate TCRBV sense primers (kindly provided by Dr. H. Dolstra, University Medical Center Nijmegen, Nijmegen, The Netherlands) and a TCRBC2 antisense primer (31). The Vα and Vβ PCR products were run on agarose gels, blotted onto Hybond filters, and hybridized with either a labeled TCRAC or a TCRBC2 probe. Positive PCR products were cloned, and plasmid DNAs from at least five independent colonies were sequenced using both the CTAB sequencing protocol and the T7 sequencing kit (Amersham Pharmacia Biotech).

Cloning of the gp100-specific MPD and 296 TCR genes and transduction of human T lymphocytes

Sequence analysis of the TCR Vα and Vβ genes used by CTL-MPD and CTL-296 allowed for the design of specific primers to amplify the full-length (fl) TCRα and β DNAs using CTL-derived cDNA as template DNA. Primers used to amplify the TCRα and TCRβ DNAs without their signal peptide sequences are as follows: fl-MPD TCR α-chain: TCRAMPD, 5′-CTC TCC ATG GAG ACT CTC CTG AAA GTG C-3′ and HCA, 5′-CTC TCT CGA GGG ATC CTC AGC TGG ACC ACA GCC GCA GC-3′; fl-MPD TCR β-chain: TCRBMPD, 5′-CTC TCC ATG GAC TCC TGG ACC TTC TGC TG-3′ and HCB, 5′-CTC TCT CGA GGG ATC GCT AGC CTC TGG AAT CCT TTC TC-3′; fl-296 TCR α-chain: TCRA296, 5′-CTC TCC ATG GCA TCC ATT CGA GCT GTA TTT-3′ and HCA; fl-296 TCR β-chain: TCRB296, 5′-CTC TCC ATG GGC CCC CAG CTC CTT GGC TAT G-3′ and HCB.

The PCR products (i.e., the TCR cDNAs) were cloned into the retroviral vector pBullet containing a heterologous signal peptide as described elsewhere (21) and checked by sequence analysis. Human T lymphocytes of healthy donors were activated with anti-CD3 mAbs and transduced with retroviruses harboring the TCRα and TCRβ transgenes. The TCR-encoding retroviruses were produced by the packaging cell line Phoenix. The transduction procedure used for primary human T lymphocytes was optimized and described by Lamers et al. (32). In short, 24-well culture plates were coated with human fibronectin fragments CH-296 (Takara Shuzo, Otsu, Japan) and pretreated with retroviral particles by centrifugation at 2400 rpm for 1 h with 2 ml retrovirus-containing supernatant per well. Next, 10^6 human T cells were centrifuged in 2 ml of fresh virus-supernatant, and cultured for 4–5 h at 37°C/5% CO_2. T cells were allowed to recover in normal T cell medium overnight before a second transduction cycle, after which cells were harvested and transferred to T25 culture flasks.

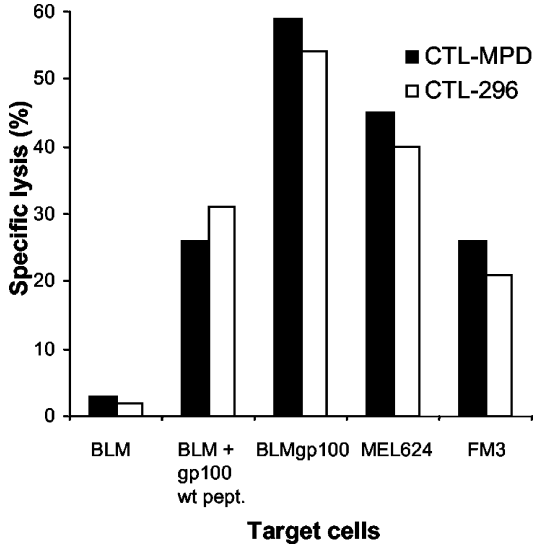

FIGURE 1. CTL-MPD and CTL-296 lyse melanoma target cells positive for the human $gp100_{280-288}$ epitope presented in the context of HLA-A2. CTL-MPD (■) and CTL-296 (□) were tested in a standard 5-h ^{51}Cr-release assay as described in *Materials and Methods*. Target cells were the $gp100^{neg}$/HLA-$A2^{pos}$ melanoma cell line BLM, with or without exogenous gp100 wt peptide (preincubation with peptide for 45 min at 37°C at a final concentration of 1 μM), BLM cells transfected with the $hgp100_{280-288}$ cDNA (BLMgp100), and the $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cell lines MEL 642 and FM3. The E:T cell ratio was 10:1.

Flow cytometry of TCR-transduced T lymphocytes

TCR-transduced T cells were analyzed for TCR and CD8α expression by flow cytometry using PE-conjugated anti-TCRVβ8 mAb (i.e., recognizing fl-MPD TCR), anti-TCRVβ14 mAb (i.e., recognizing fl-296 TCR) (both from Beckman Coulter, Marseille, France) or APC-labeled gp100/HLA-A2 tetramers, and FITC-conjugated anti-CD8 mAb (BD Biosciences, San Jose, CA), respectively. For immunostaining, $0.1-0.5 \times 10^6$ transduced cells were washed with ice-cold PBS containing 0.5% BSA and 0.02% EDTA, and incubated with mAbs on ice for 30 min or tetramers at room temperature for 1 h. For immuno double stainings, cells were first incubated with tetramers, washed, and subsequently incubated with anti-TCRVβ mAb. Upon completion of the immunostainings, cells were washed again, fixed (with 1% paraformaldehyde), and analyzed on a FACSCalibur (BD Biosciences) using CellQuest software.

Cytotoxicity assay

Cytotoxic activity of both gp100/HLA-A2-specific CTL clones and T lymphocytes that were retrovirally transduced with the fl-MPD and fl-296 TCR genes was routinely measured in a standard 5–6 h ^{51}Cr-release assay (33). Target cells were labeled with 100 μCi $Na_2{}^{51}CrO_4/10^6$ cells for at least 1 h at 37°C/5% CO_2. T2, BLM, and BSM cells were pulsed with gp100 peptides for 45 min at 37°C/5% CO_2 before cocultivation with effector T cells. To correct for NK cell activity, cold target cell inhibition assays were performed by adding nonlabeled K562 cells at the indicated cold target:hot target ratios. In experiments aimed at specific blocking of the cytolytic activities of fl-MPD and fl-296 TCR-transductants, anti-TCRVβ8 mAb (at 1 μg/ml final), anti-TCRVβ14 mAb (1 μg/ml), anti-MHC class I mAb (clone W6/32; Sera-Lab, Crawley Down, U.K.) (10 μg/ml), or mouse γ globulin (mIg; Jackson ImmunoResearch Laboratories, West Grove, PA) (10 μg/ml) was added at the onset of the cytotoxicity assay. Cytolytic efficiencies of parental CTL and TCR transductants were assayed by pulsing T2 cells with various amounts of the gp100 wt peptide, whereas peptide fine specificities were determined by loading T2 cells with a series of gp100 peptide mutants (all at 1 μM final) before cytotoxicity assays. Percentage of specific cytolysis, i.e., specific ^{51}Cr-release, was calculated as described previously (34).

TNF-α production

To quantify TNF-α production by TCR-transduced T lymphocytes after Ag-specific stimulation, 6×10^4 T cells were cultured in the presence of 2×10^4 tumor cells or peptide-pulsed T2 cells for 18 h. As a positive control, TCR-transduced T lymphocytes were stimulated with PHA and PMA. Supernatants were harvested and levels of TNF-α were measured by standard ELISA (Central Laboratory for Blood Transfusions, Amsterdam, The Netherlands) according to the manufacturer's instructions.

Results

Two distinct anti-gp100 CTL use a highly similar TCRβ region

We generated an HLA-A2-restricted $gp100_{280-288}$-specific CTL by stimulating PBL from a healthy donor with autologous peptide-pulsed DC (5). Limiting dilution subsequently yielded the CTL clone MPD which showed a cytotoxic reactivity against peptide-pulsed and native $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cells (Fig. 1) that was identical with the reactivity pattern of the CTL bulk culture. No lysis of the $gp100^{neg}$/HLA-$A2^{pos}$ melanoma cell line BLM was observed, whereas BLM cells pulsed with the gp100 wt peptide or transfected with human gp100-encoding cDNA (BLMgp100) were efficiently lysed by CTL-MPD (Fig. 1). In addition, the $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cell lines MEL624 and FM3 were also lysed by this CTL clone (Fig. 1). PCR-based typing of the TCR$\alpha\beta$ genes of CTL-MPD and sequence analysis of

```
AV29s1                -N-                                    JA49                    AC

TCRα MPD
AAC TAC TTC TGT  GGC ACA GAG ACG AAC ACC GGT AAC            CAG TTC TAT TTT GGG ACA GGG ACA AGT TTG ACG GTC ATT CCA AAT  ATC CAG
 T   Y   F   C   G   T   E   T   N   T   G   N   -   -   -  Q   F   Y   F   G   T   G   T   S   L   T   V   I   P   N   I   Q
 V   Y   F   C   A   A   S   T   S   G   G   T   S   Y   G  K   L   T   F   G   Q   G   T   I   L   T   V   H   P   N   I   Q
GTC TAC TTC TGT  GCA GCA AGT ACT TCG GGT GGT ACT AGC TAT GGA AAG CTG ACA TTT GGA CAA GGG ACC ATC TTG ACT GTC CAT CCA AAT  ATC CAG
TCRα 296

AV8s1                 -N-                                    JAJ                     AC


BV8s2            -N-   -D1s1-    -N-                          JB2s1                   BC2

TCRβ MPD
TTC TGT GCC AGC AGT  TTG  GGG CGG  TAC AAT  GAG CAG TTC TTC GGG CAA GGG ACA CGG CTC ACC GTG CTA  GAG GAC
 F   C   A   S   S   L   G   R   Y   N   E   Q   F   F   G   Q   G   T   R   L   T   V   L   E   D
 F   C   A   S   S   L   G   S   S   Y   E   Q   Y   F   G   P   G   T   R   L   T   V   T   E   D
TTC TGT GCC AGC AGT  TTG  GGG AGC  TCC TAC  GAG CAG TAC TTC GGG CCG GGC ACC AGG CTC ACG GTC ACA  GAG GAC
TCRβ 296

BV14s1           -N-   -D1s1-    -N-                          JB2s7                   BC2
```

FIGURE 2. Alignment of TCR CDR3 regions of CTL-MPD and CTL-296. Nucleotide and amino acid sequence alignment of the CDR3 regions of the TCRα as well as β-chains of both CTL-MPD and CTL-296. Identical amino acids are indicated by a vertical line. CDR3 regions were defined according to Chothia et al. (35), and TCR variable segments were designated according to Arden et al. (36).

cDNA clones revealed that the TCR α- and β-chains of CTL-MPD, respectively comprise the segments AV29s1/JA49 and BV8s2/D1s1/JB2s1, respectively (Fig. 2). In fact, sequence analysis of 17 different CTL clones showed that they all express the same TCR β-chain and thus are derived from one parental CTL (data not shown).

Interestingly, homology searches revealed the existence of an almost identical TCR β-chain of another HLA-A2 restricted anti-$gp100_{280-288}$ CTL clone, i.e., CTL-296/147, which was generated by stimulating PBL from an HLA-$A2^{pos}$ melanoma patient with autologous $gp100^{pos}$ tumor cells (24). The overall cytotoxic response of this CTL appeared to be identical with that of CTL-MPD (Fig. 1). Sequence analysis performed on cDNA clones from this melanoma patient-derived CTL-296 showed that its TCR$\alpha\beta$ consists of the segments AV8s1/JAJ and BV14s1/D1s1/JB2s7 (Fig. 2). Although both CTL-MPD and CTL-296 clearly use different Vβ and Jβ gene segments, the TCRβ complementarity-determining region (CDR)3 of both clones are strikingly homologous at the amino acid level, and in fact identical in size (Fig. 2). The resemblance between the TCRα CDR3 regions of both CTL clones is less pronounced.

CTL-MPD and CTL-296 differ in their gp100 peptide fine specificity

To determine the cytolytic efficiencies of CTL-MPD and CTL-296, T2 target cells were pulsed with various amounts of the gp100 wt peptide and specific lysis was measured in a ^{51}Cr-release assay. From Fig. 3*A*, it is clear that the amount of gp100 wt peptide required to obtain 50% of the maximum lysis of peptide-pulsed T2 cells (ED_{50}) was similar for both CTL-MPD and CTL-296 (ED_{50} = 100 and 50 pM peptide, respectively). A control HLA-A2-binding peptide was not recognized by both CTL, even at higher concentrations (data not shown). These findings show that both CTL display similar cytolytic efficiencies toward gp100 wt peptide-loaded target cells.

Next, we studied the importance of each individual amino acid residue in the $gp100_{280-288}$ peptide with respect to TCR: peptide/MHC interactions. A series of peptide analogs was synthesized in which the native amino acid of the gp100 wt peptide, i.e., YLEPGPVTA, was substituted for an alanine or glycine. The peptides were first assayed for their binding to HLA-A2 via both an indirect binding assay, using the Ag-processing defective cell line T2 (29) and a peptide binding competition assay using JY cells (30). Both assays showed that only amino acid substitutions at positions 2 and 9 have a drastic effect on the peptide binding to HLA-A2 molecules (Table I), consistent with the notion that the amino acids at positions 2 and 9 are anchor residues involved in HLA-A2 binding (37). Subsequently, the cytotoxic CTL responses toward T2 cells loaded with the various gp100 peptide mutants were assayed to determine the functional peptide requirements of the TCR$\alpha\beta$ of both CTL-MPD and CTL-296. It is apparent from Fig. 4*A* that gp100 peptide mutants with substitutions at the anchor positions still initiated a cytotoxic response by both CTL clones, despite their poor binding to HLA-A2. This is in line with the observation that only a few peptide/MHC complexes on target cells are sufficient to trigger CTL responses (38). However, the gp100 peptide with an alanine substitution at position 3 did not trigger lysis by either CTL clone (see Fig. 4*A*), indicating that the glutamic acid at position 3 of the gp100 peptide is crucial for TCR recognition. Furthermore, no substitutions were allowed at positions 1 and 4 of the gp100 peptide for peptide-mediated lysis by CTL-MPD. In contrast, peptide analogs with substitutions at positions 1 and 4 were still capable of sensitizing T2 cells for lysis by CTL-296 (Fig. 4*A*). Peptide titration experiments confirmed that the alanine 1 and 4 mutants were as efficient as the gp100 wt peptide with respect to peptide-induced cytolysis by CTL-296 but not CTL-MPD (data not shown). Taken together, the CTL-MPD displays a peptide requirement that is more stringent than that of CTL-296.

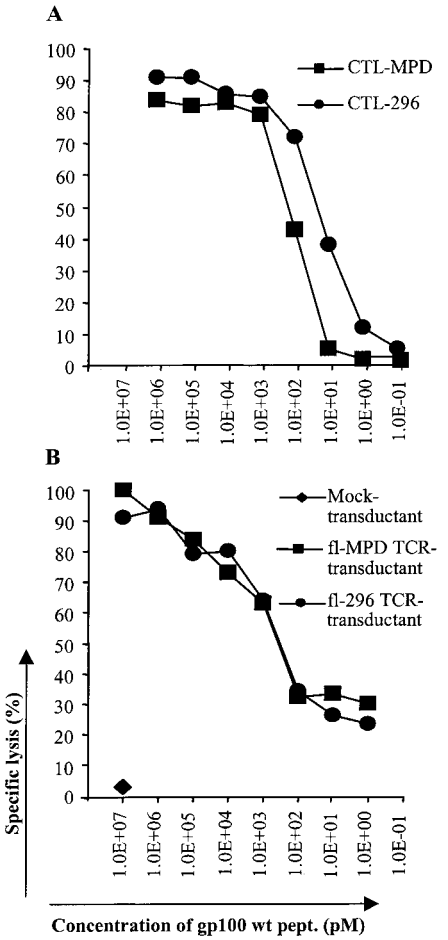

FIGURE 3. Cytolytic efficiencies of CTL-MPD and CTL-296 are preserved following transfer of engineered TCR$\alpha\beta$ genes into primary human T lymphocytes. *A*, CTL-MPD and CTL-296 show a similar efficiency in gp100 wt peptide-induced cytotoxicity. CTL-MPD (■) and CTL-296 (●) were tested in a 5-h ^{51}Cr-release assay using T2 cells, preincubated with various amounts of gp100 wt peptide for 45 min at 37°C, as target cells. The E:T cell ratio was 10:1. The peptide concentration corresponding to 50% of the maximum lysis (ED_{50}), used as a measure of cytolytic efficiency, was 100 and 50 pM for the CTL-MPD and CTL-296, respectively. *B*, Cytolytic efficiencies of fl-MPD and fl-296 TCR^{pos} human T lymphocytes are in the same range as the cytolytic efficiencies of the parental CTL clones. Human T cells transduced with the fl-MPD and fl-296 TCR genes (■ and ●, respectively) and mock-transduced human T lymphocytes (◆) were tested in a 6-h ^{51}Cr-release assay. T2 cells, preincubated with various amounts of gp100 wt peptide, were used as target cells. The E:T cell ratio was 15:1. Cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The peptide concentration corresponding to the ED_{50} of both fl-MPD TCR and fl-296 TCR-transductants was 300 pM. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

TCR gene transfer into primary human T lymphocytes

The existence of two gp100-specific CTL clones with distinct gp100 peptide requirements allowed us to investigate the preservation of peptide fine specificity of native TCRs following TCR

Table I. *HLA-A2 binding capacity of gp100 peptide mutants*

Peptide	Stabilization Assay with T2 Cells (μM)[a] 50	25	Competition Assay with JY Cells[b]
WMAFKERKV[c]	0.00	0.00	
FLPSDFFPSV[d]	1.76	1.34	0.6
YLEPGPVTA[e]	1.25	1.30	9.6
A1	0.87	0.73	53.3
A2	0.00	0.00	41000.0
A3	1.29	1.54	2.3
A4	1.36	0.82	3.9
A5	1.56	1.35	9.3
A6	0.98	1.11	14.6
A7	1.30	1.51	11.5
A8	1.61	1.70	6.5
G9	0.13	0.09	3367.3

[a] HLA-A2 stabilization by peptides was analyzed by incubating the processing defective cell line T2 with the indicated peptide concentrations. Values indicate fluorescence index: (experimental mean fluorescence/background mean fluorescence) − 1. See *Materials and Methods* for details.

[b] Binding capacity for peptides was determined using the competition-based assay with JY cells. The binding capacity of a peptide is expressed as the concentration required to inhibit 50% of binding of a FL-labeled reference peptide (IC_{50} in micromolars). See *Materials and Methods* for details.

[c] WMAFKERKV is a non-HLA-A2-binding peptide.

[d] FLPSDFFPSV is used as an HLA-A2-binding reference peptide in the competition assay.

[e] YLEPGPVTA is the $gp100_{280-288}$ wt peptide. gp100 peptide mutants are encoded as described in the legend to Fig. 4*A*.

gene transfer into human T lymphocytes. To this end, we isolated fl TCR$\alpha\beta$ cDNAs from both CTL, engineered them for retroviral expression using the vector pBullet (21) and the packaging cell line Phoenix, and transferred them into primary human T lymphocytes. It is important to note that the used retroviral transduction protocol (described in detail in Ref. 32) allows for high transduction efficiencies of primary human T lymphocytes that can then be directly assayed in functional tests and does not necessitate subsequent enrichment or cloning of TCR-transduced T lymphocytes.

Approximately 50% of the human T lymphocytes stably expressed the fl-MPD and fl-296 TCR transgenes, as determined by flow cytometry using anti-TCRVβ8 and anti-TCRVβ14 mAbs, respectively (Fig. 5*A*). Primary human T cell transductants were checked weekly for expression of the introduced TCR chains by flow cytometry, and TCR expression remained stable for a period of at least 3 mo in continuous culture. Expression of CD8 was always >50% (data not shown). In addition, the TCR-transduced human T lymphocytes specifically bound $gp100_{280-288}$/HLA-A2 tetramers (Fig. 5*B*), whereas the binding of an irrelevant EBV/HLA-A2 tetramer was negligible. Interestingly, when analyzing the TCRβ and gp100/HLA-A2 tetramer stainings at the single-cell level, we observed that of T cells expressing the introduced TCRβ, only 50–60% are able to bind the gp100/HLA-A2 tetramer. Results were confirmed when looking within the CD8-positive fraction of the T cell transductants.

As shown in Fig. 6*A*, the fl-MPD and fl-296 TCR-transduced human T lymphocytes lysed the gp100 wt peptide-loaded HLA-$A2^{pos}$ B-LCL BSM and the $gp100^{pos}$/HLA-$A2^{pos}$ melanoma cell lines BLMgp100, FM3, and MEL624 very efficiently, whereas the HLA-$A2^{pos}$ BLM or BSM cells loaded with an irrelevant EBV peptide were not lysed. Mock-transduced human T lymphocytes did not lyse any of the tumor cell lines. The cytolytic responses of the fl-MPD and fl-296 TCR^{pos} T lymphocytes against the MEL624 melanoma cell line were almost completely inhibited by anti-TCRVβ mAb (CTL-MPD: anti-TCRVβ8 mAb, and CTL-296: anti-TCRVβ14 mAb) and anti-MHC class I mAb (Fig. 6*B*). mIg as a

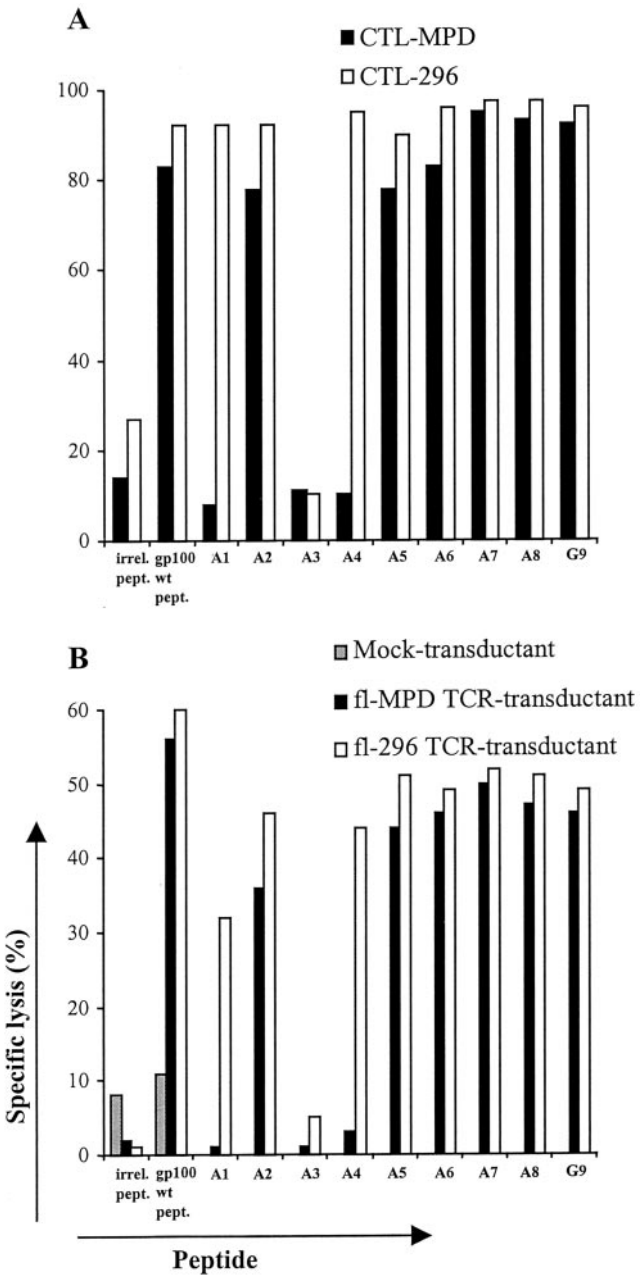

FIGURE 4. Differences in peptide fine specificities between CTL-MPD and CTL-296 are preserved following transfer of engineered TCR$\alpha\beta$ genes into primary human T lymphocytes. *A*, CTL-MPD and CTL-296 differ in their gp100 peptide fine specificity. CTL-MPD (■) and CTL-296 (□) were tested in a 5-h ^{51}Cr-release assay. T2 cells were preincubated with gp100 peptide mutants (at 1 μM final, see *Materials and Methods* for details), and used as target cells. As a negative control an irrelevant HLA-A2-binding $gp100_{154-162}$-peptide (irrel. pept.) and as a positive control the $gp100_{280-288}$ peptide (gp100 wt pept.) were used. gp100 peptide mutants are encoded as follows: the letter indicates an alanine (A) or glycine (G) substitution, and the number corresponds to the amino acid position of the substituted amino acid relative to wt peptide. The E:T cell ratio was 10:1. *B*, fl-MPD and fl-296 TCR-transduced human T lymphocytes show gp100 peptide fine specificities identical with those of the parental CTL clones. Human T cells transduced with the fl-MPD and fl-296 TCR genes (■ and □, respectively) and mock-transduced human T lymphocytes (▨) were tested in a 6-h ^{51}Cr-release assay using T2 cells, pulsed with gp100 peptide mutants, as target cells. The E:T cell ratio was 15:1. Cold K562 cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

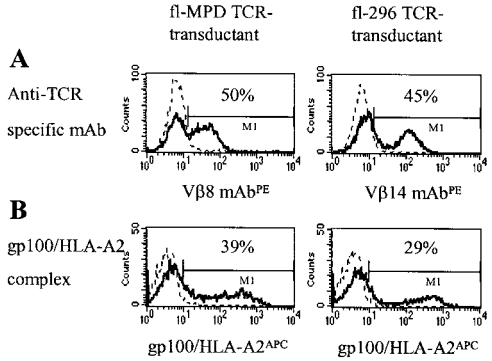

FIGURE 5. Cell surface expression of gp100-specific fl-MPD and fl-296 TCR on transduced primary human T lymphocytes. *A*, Human T lymphocytes retrovirally transduced with genes encoding gp100-specific TCR$\alpha\beta$ show cell surface expression of the TCR transgenes. T lymphocytes transduced with the fl-MPD and fl-296 TCR$\alpha\beta$ genes specific for gp100 (solid lines) were labeled with the PE-conjugated TCR family type-specific anti-TCRVβ8 and anti-TCRVβ14 mAbs, respectively, and analyzed by flow cytometry. *B*, T lymphocytes transduced with gp100-specific TCR genes specifically bind gp100 peptide/MHC complexes. The gp100 TCR transductants described in the legend to *A* were labeled with APC-conjugated $gp100_{280-288}$/HLA-A2 tetramers before flow cytometric analysis (solid lines). Mock-transduced human T lymphocytes (dotted lines) served as negative controls. Marker M1 was set in the corresponding histogram of mock-transduced T cells at a 5% expression level, and percentages given reflect the fraction of stained TCR transductants relative to M1. The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. Three additional donors provided similar data. The average transduction efficiencies were 49% (range: 27–60%) and 46% (range: 39–54%) for the anti-TCRVβ8 and anti-TCRVβ14 mAbs, respectively.

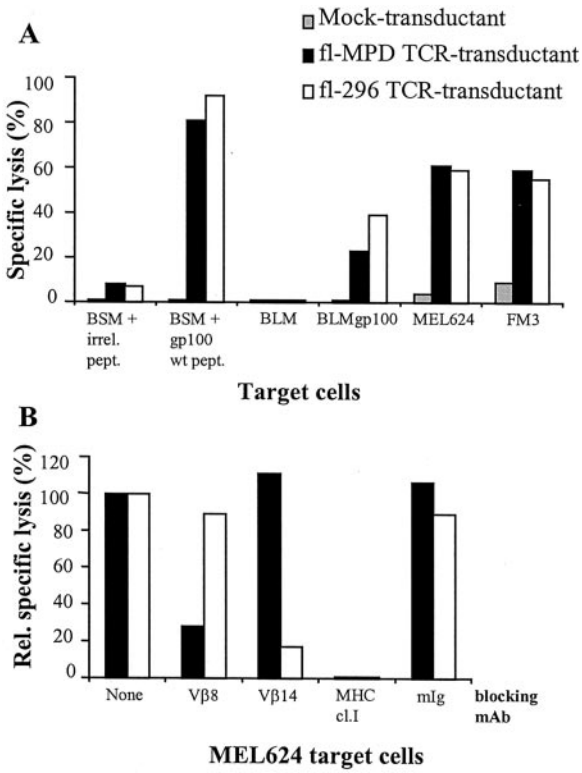

FIGURE 6. Human T lymphocytes transduced with fl-MPD and fl-296 TCR genes show Ag-specific cytolysis of $gp100^{pos}$/$HLA\text{-}A2^{pos}$ tumor cells. *A*, fl-MPD and fl-296 TCR^{pos} human T lymphocytes lyse melanoma cells positive for the human $gp100_{280-288}$ peptide presented in the context of HLA-A2. Human T lymphocytes transduced with the fl-MPD and fl-296 TCR genes (■ and □, respectively) and mock-transduced human T lymphocytes (▨) were tested in a 6-h ^{51}Cr-release assay. The following target cells were used: the $HLA\text{-}A2^{pos}$ B-LCL BSM, pulsed with either gp100 wt peptide or an irrelevant HLA-A2-binding EBV peptide (both at 10 μM final), BLM, BLMgp100, FM3, and MEL624 (as described in the legend to Fig. 1). The E:T cell ratio was 15:1. Cold K562 target cells were added to ^{51}Cr-labeled target cells at a ratio of 30:1. The results reflect fl-TCR transductions of primary human T lymphocytes of one representative donor of two. *B*, The cytotoxic response of fl TCR-transduced human T lymphocytes toward $gp100^{pos}$ melanoma cells is specifically blocked by anti-TCRVβ and MHC class I mAbs. Cytotoxicity assays were performed with fl-MPD and fl-296 TCR^{pos} T lymphocytes as effector cells (■ and □, respectively), and the $gp100^{pos}$/$HLA\text{-}A2^{pos}$ melanoma cell line MEL624 as target cells under the same conditions as described above but in the presence of anti-TCRVβ8 mAb (at 1 μg/ml final), anti-TCRVβ14 mAb (1 μg/ml), anti-MHC class I mAb (10 μg/ml), or mIg (10 μg/ml). Immune specific lysis was expressed relative to the lysis of MEL624 in the absence of mAbs (set to 100%: corresponding to 41% specific lysis for both the fl-MPD and fl-296 TCR transductants). The results shown represent fl-TCR transductions of primary human T lymphocytes of one donor. A second donor provided similar data.

control Ab did not block the cytolytic responses of either TCR transductant (Fig. 6*B*). In addition, the fl-MPD and fl-296 TCR-transduced human T lymphocytes were also specifically triggered to produce TNF-α in response to peptide-loaded and native $gp100^{pos}$ tumor target cells (Table II).

Next to Ag reactivity, we studied the cytolytic efficiencies of the fl-MPD and fl-296 TCR^{pos} human T lymphocytes by measuring specific lysis toward T2 target cells loaded with several dilutions of the gp100 wt peptide. The ED_{50} of gp100 wt peptide is ~300 pM for both fl-MPD and fl-296 TCR^{pos} human T lymphocytes (Fig. 3*B*), which is in the same range as the cytolytic efficiencies of the parental CTL-MPD and CTL-296 clones (ED_{50} = 100 and 50 pM, respectively; Fig. 3*A*). T2 cells loaded with a high concentration of an irrelevant HLA-A2-binding EBV peptide (10 μM final) were not lysed by the TCR-transduced human T lymphocytes (data not shown), and mock-transduced human T lymphocytes did not lyse T2 cells loaded with gp100 wt peptide (at 10 μM final) (Fig. 3*B*).

In total, the TCR-transduced human T lymphocytes show specific immune responses to $gp100^{pos}$/$HLA\text{-}A2^{pos}$ target cells, and exhibit similar cytolytic efficiencies as their corresponding parental CTL clones.

Peptide requirement of gp100-specific TCR-transduced primary human T lymphocytes is identical with that of parental CTL

The preservation of not only the cytotoxic potency but also the peptide fine specificity of CTL by TCR gene transfer is crucial to the potential use of TCR genes for immunotherapy. To address this issue, the peptide fine specificities of the fl-MPD and fl-296 TCR transductants were determined in a ^{51}Cr-release assay with T2 cells pulsed with a series of gp100 peptide analogs (as described in the legend to Fig. 4*A*). The gp100 peptide with an alanine substitution at position 3 did not trigger lysis by either TCR transductant (Fig. 4*B*). In addition to position 3, no substitutions were allowed at positions 1 and 4 for peptide-mediated lysis by the fl-MPD TCR^{pos} T lymphocytes, whereas these latter peptide analogs were still capable of sensitizing T2 cells for lysis by fl-296 TCR^{pos} T lymphocytes (Fig. 4*B*). We conclude from these experiments that the TCR transductants show gp100 peptide fine specificities that are identical with those of the parental CTL (compare Fig. 4, *A* and *B*).

Table II. *fl-MPD and fl-296 TCR-transduced T lymphocytes produce TNF-α after stimulation with gp100^{pos}/HLA-A2^{pos} target cells*

Target Cells	fl-MPD TCR Transductants	fl-296 TCR Transductants
None	0[a]	2
BLM	8	4
BLMgp100	378	716
FM3	141	329
MEL624	120	223
T2 + irrel. pept.	40	14
T2 + gp100 wt pept.	365	810

[a] fl-MPD and fl-296 TCR^{pos} human T lymphocytes were cocultivated for 18 h with BLM, BLMgp100, FM3, MEL624, and T2 cells, pulsed with irrelevant HLA-A2-binding EBV peptide (irrel. pept.) or gp100 wt peptide (wt pept.) (both at 1 μM final), at an E:T cell ratio of 3:1. Cell-free supernatants were harvested and analyzed in a TNF-α ELISA. Levels of TNF-α are expressed in picograms per milliliter. Mock-transduced T lymphocytes did not produce TNF-α after stimulation with any target cell. PHA/PMA-stimulated T lymphocytes produced >1500 pg/ml TNF-α.

Discussion

This paper reports on the molecular and functional characterization of two distinct CTL clones each specific for the native melanoma differentiation Ag gp100 presented by HLA-A2, and the preservation of specific Ag reactivity and peptide fine specificity following transfer of engineered TCR genes into primary human T lymphocytes. The CTL clones described in this paper have different origins, one CTL being derived from a healthy individual using peptide-pulsed DC (i.e., CTL-MPD) and the other derived from a melanoma patient using autologous melanoma cells as APC (i.e., CTL-296), and express TCRα- and β-chains using different TCRVα and Vβ gene segments. The two CTL clones are both specific for the same gp100$_{280-288}$ epitope but clearly differ in their response to amino acid substitutions in this gp100 epitope. fl TCRαβ genes of either CTL clone, when engineered for gene transfer into primary human T lymphocytes, completely preserve the observed gp100-specific Ag reactivity and cytolytic efficiency. Moreover, the characterized peptide fine specificities of the two native anti-gp100 TCRs were also functionally exhibited by T lymphocytes transduced with the fl TCRs.

The TCR chains of the gp100-specific CTL-MPD and CTL-296 have strictly homologous CDR3β but not CDR3α regions (see Fig. 2), implying the selection of a specific amino acid sequence in the CDR3β region. The same pattern of sequence and size conservation of the CDR3 regions of the TCRα- and β-chains was observed in a panel of CTL with the same Ag specificity by Moss and Bell (39). These findings fit the idea that the TCRα- and β-chains contribute differently to the recognition of the peptide/MHC complex. This notion is further supported by the crystal structure of a TCR bound to a viral peptide/HLA-A2 complex (40). Although several studies show that conservation of sequence and size of CDR3β of CTL is not an absolute determinant of Ag recognition, TCR CDR3 regions do appear to govern peptide fine specificity of CTL (41, 42).

Analysis of the cytotoxic responses of CTL-MPD and CTL-296 showed that both CTL clones have an identical Ag reactivity toward gp100^{pos}/HLA-A2^{pos} melanoma target cells (see Fig. 1). Furthermore, both CTL clones lyse gp100 wt peptide-pulsed target cells with efficiencies in the picomolar range (see Fig. 3*A*), suggesting that the TCR of both CTLs bind the gp100 peptide/HLA-A2 complex on target cells with similar affinities. Nevertheless, the specificities of CTL-MPD and CTL-296 for gp100 peptides with amino acid substitutions are strikingly different (see Fig. 4*A*). The CTL responses toward gp100 peptide mutants indicate that the amino acids in the gp100 epitope important for TCR: peptide/MHC interaction of either CTL clone overlap but are not identical. Both parental CTL clones are equally sensitive to a substitution of the glutamic acid at amino acid position 3 of the gp100 peptide for alanine, but only CTL-MPD is sensitive to alanine substitutions at amino acid positions 1 and 4. Because the tested amino acid changes do not affect binding of the gp100 peptide to HLA-A2 (see Table I), a peptide mutant may be incorrectly positioned in the MHC molecule and result in inadequate TCR recognition and CTL response.

Our observation that peptide-pulsed DCs can induce bona fide CTL clones capable of lysing melanoma tumor cells endogenously processing and presenting the tumor Ag in vitro supports a treatment strategy of melanoma patients with DC pulsed with peptides derived from melanoma-associated Ags, which we and others currently employ. The molecular analysis of the TCRα- and β-chains expressed by gp100-reactive CTLs allows for TCR gene transfer into primary human T lymphocytes, a possible alternative to immunotherapy protocols. The feasibility of transferring fl as well as chimeric two-chain and single-chain TCR genes to impose an antitumor or antivirus specificity onto T cells has been reported by us and others (17, 18, 20–23, 43–45). The present study demonstrates for the first time that TCR gene transfer not only preserves peptide specificity, but even peptide fine specificity, which is crucial to the use of TCR genes for immunogene therapy. Important to TCR gene transfer studies, but often unaccounted for, is the efficiency which one can obtain to introduce TCR genes into primary human T lymphocytes. We used a retroviral transduction protocol that results in high levels of surface expression of TCR chains on human T lymphocytes (~50% of bulk cultures of transduced T lymphocytes expressed the appropriate TCR β-chain, and 35% of these bulk cultures bound gp100$_{280-288}$/HLA-A2 tetramers; see Fig. 5). This is in contrast to previous reports that show only poor retroviral transduction efficiencies of TCR genes, necessitating antibiotic selection, enrichment for CD8-positive T cells or even cloning to obtain a tumor or virus-specific response of retargeted T cells (17–19, 44). Parameters that greatly affect retroviral transduction efficiencies, such as transduction-mediating agents and type of retroviral vector, have been carefully optimized by our group and others, allowing for gene transfer into primary human T lymphocytes with high efficiency (21, 34, 46) and at clinical scale meeting the criteria of Good Medical Practice (32).

Retargeted T cells were analyzed functionally for their lytic activity and ability to produce TNF-α in response to the same panel of target cells as used to analyze the CTL clones (see Fig. 6*A* and Table II). The Ag reactivities of the TCR transductants are identical with those of the parental CTL clones (compare Figs. 1 and 6*A*), and were confirmed by blocking studies with family type-specific anti-TCR Vβ as well as anti-MHC class I Abs (see Fig. 6*B*). Subsequent peptide titration experiments revealed that the cytolytic efficiencies of the T lymphocyte transductants are in the same picomolar range as those of the parental CTL clones (compare Fig. 3, *A* and *B*). Importantly, primary human T lymphocytes positive for either gp100/HLA-A2-specific TCR show cytolytic activities in response to a large panel of peptide mutants that are identical with those of the parental CTL clones (compare Fig. 4, *A* and *B*). Moreover, production levels of TNF-α of the TCR transductants in response to the gp100 peptide mutants reflect the peptide fine specificity observed in the cytotoxicity assays (data not shown). The observation that TCRs engineered for gene transfer and introduced into primary human T lymphocytes completely preserve the peptide fine specificities of the native TCRs implies that the exogenous TCR genes, in particular their CDR3 regions, do properly fold when expressed on T cell membranes.

The preservation of the peptide fine specificity of parental CTL by transfer of fl TCR genes makes TCR transductants promising tools for immunogene therapy. However, an important issue that needs to be addressed regarding the clinical use of such TCR gene-transduced T lymphocytes is the possible formation of new, potentially autoimmune TCR$\alpha\beta$ heterodimers comprising exogenous and endogenous TCR chains. Flow cytometry at the single-cell level shows that following transfer of both TCRα and β genes only a fraction (i.e., 50–60%) of CD8-positive T cells expressing the introduced TCR β-chain are able to bind the gp100/HLA-A2 tetramer. These findings agree with the observation by Stanislawski and colleagues (22) that upon transfer of MDM-2-specific TCR$\alpha\beta$ genes into primary human T cells only 30–50% of T cells positive for the introduced TCRβ were able to bind the MDM-2-specific tetramer, and suggest that the introduced and surface-expressed TCRβ chains do pair with endogenous TCR α-chains. The transfer of chimeric TCR genes linked to genes encoding signal transduction molecules, such as CD3ζ, has proven advantageous in this respect since it facilitates pairing between two CD3ζ-containing proteins (21, 47), thereby maximizing the expression of the introduced TCR and reducing the risk that alternative TCR complexes are formed. Another advantage of tumor-specific receptors chimerized to CD3ζ or other signaling molecules is that they bypass TCR-mediated proximal signaling events (48), which are often defective in cancer patients (49, 50). Recently, we have made chimeric genes comprising the Vα and Vβ domains of the fl-MPD and fl-296 TCR genes coupled to various signaling molecules, such as CD3ζ and Fc(ϵ)RIγ, which are currently tested for their efficiency in T cell retargeting.

Taken together, in this report gp100-specific TCR, derived from two different CTL, have been molecularly analyzed and functionally introduced into primary human T lymphocytes without loss of the Ag reactivity and peptide fine specificity. These results confirm the notion that TCR gene transfer holds promise for immunogene therapy.

Acknowledgments

We thank Drs. H. Dolstra, P. van der Elsen, and T. Logtenberg for providing PCR primers; J. W. Drijfhout for peptide synthesis; H. Spits for providing $gp100_{280-288}$/HLA-A2 tetramers; H. Zarour and P. van der Bruggen for the CTL-296 clone; and M. W. J. Schreurs, J. L. M. Vissers, and A. B. H. Bakker for fruitful discussions.

References

1. Renkvist, N., C. Castelli, P. F. Robbins, and G. Parmiani. 2001. A listing of human tumor antigens recognized by T cells. *Cancer Immunol. Immunother. 50:3.*
2. Rosenberg, S. A. 2001. Progress in human tumour immunology and immunotherapy. *Nature 411:380.*
3. Kawakami, Y., S. Eliyahu, C. H. Delgado, P. F. Robbins, K. Sakaguchi, E. Appella, J. R. Yannelli, G. J. Adema, T. Miki, and S. A. Rosenberg. 1994. Identification of a human melanoma antigen recognized by tumor-infiltrating lymphocytes associated with in vivo tumor rejection. *Proc. Natl. Acad. Sci. USA 91:6458.*
4. Cox, A. L., J. Skipper, Y. Chen, R. A. Henderson, T. L. Darrow, J. Shabanowitz, V. H. Engelhard, D. F. Hunt, and C. L. Slingluff, Jr. 1994. Identification of a peptide recognized by five melanoma-specific human cytotoxic T cell lines. *Science 264:716.*
5. Bakker, A. B., G. Marland, A. J. de Boer, R. J. Huijbens, E. H. Danen, G. J. Adema, and C. G. Figdor. 1995. Generation of antimelanoma cytotoxic T lymphocytes from healthy donors after presentation of melanoma-associated antigen-derived epitopes by dendritic cells in vitro. *Cancer Res. 55:5330.*
6. Tsai, V., S. Southwood, J. Sidney, K. Sakaguchi, Y. Kawakami, E. Appella, A. Sette, and E. Celis. 1997. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells. *J. Immunol. 158:1796.*
7. Freudenthal, P. S., and R. M. Steinman. 1990. The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc. Natl. Acad. Sci. USA 87:7698.*
8. Larsen, C. P., S. C. Ritchie, R. Hendrix, P. S. Linsley, K. S. Hathcock, R. J. Hodes, R. P. Lowry, and T. C. Pearson. 1994. Regulation of immunostimulatory function and costimulatory molecule (B7-1 and B7-2) expression on murine dendritic cells. *J. Immunol. 152:5208.*
9. Flechner, E. R., P. S. Freudenthal, G. Kaplan, and R. M. Steinman. 1988. Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction. *Cell. Immunol. 111:183.*
10. Collins, R. H., Jr., O. Shpilberg, W. R. Drobyski, D. L. Porter, S. Giralt, R. Champlin, S. A. Goodman, S. N. Wolff, W. Hu, C. Verfaillie, et al. 1997. Donor leukocyte infusions in 140 patients with relapsed malignancy after allogeneic bone marrow transplantation. *J. Clin. Oncol. 15:433.*
11. Heslop, H. E., and C. M. Rooney. 1997. Adoptive cellular immunotherapy for EBV lymphoproliferative disease. *Immunol. Rev. 157:217.*
12. Rosenberg, S. A. 1999. A new era of cancer immunotherapy: converting theory to performance. *CA Cancer J. Clin. 49:70, 65.*
13. Hanson, H. L., D. L. Donermeyer, H. Ikeda, J. M. White, V. Shankaran, L. J. Old, H. Shiku, R. D. Schreiber, and P. M. Allen. 2000. Eradication of established tumors by $CD8^+$ T cell adoptive immunotherapy. *Immunity 13:265.*
14. Theobald, M., J. Biggs, J. Hernandez, J. Lustgarten, C. Labadie, and L. A. Sherman. 1997. Tolerance to p53 by A2.1-restricted cytotoxic T lymphocytes. *J. Exp. Med. 185:833.*
15. Bolhuis, R. L., and J. W. Gratama. 1998. Genetic re-targeting of T lymphocyte specificity. *Gene Ther. 5:1153.*
16. Bolhuis, R. L., R. A. Willemsen, and J. W. Gratama. 2000. Clinical applications of redirected cytotoxicity. In *Cytotoxic cells: Basic Mechanisms and Medical Applications*. M. V. Sitkovsky and P. A. Henkart, eds. Lippincott Williams & Wilkins, Philadelphia, p. 423.
17. Clay, T. M., M. C. Custer, J. Sachs, P. Hwu, S. A. Rosenberg, and M. I. Nishimura. 1999. Efficient transfer of a tumor antigen-reactive TCR to human peripheral blood lymphocytes confers anti-tumor reactivity. *J. Immunol. 163:507.*
18. Cooper, L. J., M. Kalos, D. A. Lewinsohn, S. R. Riddell, and P. D. Greenberg. 2000. Transfer of specificity for human immunodeficiency virus type 1 into primary human T lymphocytes by introduction of T-cell receptor genes. *J. Virol. 74:8207.*
19. Fujio, K., Y. Misaki, K. Setoguchi, S. Morita, K. Kawahata, I. Kato, T. Nosaka, K. Yamamoto, and T. Kitamura. 2000. Functional reconstitution of class II MHC-restricted T cell immunity mediated by retroviral transfer of the $\alpha\beta$ TCR complex. *J. Immunol. 165:528.*
20. Orentas, R. J., S. J. Roskopf, G. P. Nolan, and M. I. Nishimura. 2001. Retroviral transduction of a T cell receptor specific for an Epstein-Barr virus-encoded peptide. *Clin. Immunol. 98:220.*
21. Willemsen, R. A., M. E. Weijtens, C. Ronteltap, Z. Eshhar, J. W. Gratama, P. Chames, and R. L. Bolhuis. 2000. Grafting primary human T lymphocytes with cancer-specific chimeric single chain and two chain TCR. *Gene Ther. 7:1369.*
22. Stanislawski, T., R. H. Voss, C. Lotz, E. Sadovnikova, R. A. Willemsen, J. Kuball, T. Ruppert, R. L. Bolhuis, C. J. Melief, C. Huber, et al. 2001. Circumventing tolerance to a human MDM2-derived tumor antigen by TCR gene transfer. *Nat. Immunol. 2:962.*
23. Kessels, H. W., M. C. Wolkers, M. C. van den Boom, M. A. van der Valk, and T. N. Schumacher. 2001. Immunotherapy through TCR gene transfer. *Nat. Immunol. 2:957.*
24. Zarour, H., C. De Smet, F. Lehmann, M. Marchand, B. Lethe, P. Romero, T. Boon, and J. C. Renauld. 1996. The majority of autologous cytolytic T-lymphocyte clones derived from peripheral blood lymphocytes of a melanoma patient recognize an antigenic peptide derived from gene Pmel17/gp100. *J. Invest. Dermatol. 107:63.*
25. van de Griend, R. J., B. A. Van Krimpen, S. J. Bol, A. Thompson, and R. L. Bolhuis. 1984. Rapid expansion of human cytotoxic T cell clones: growth promotion by a heat-labile serum component and by various types of feeder cells. *J. Immunol. Methods 66:285.*
26. Bakker, A. B., M. W. Schreurs, A. J. de Boer, Y. Kawakami, S. A. Rosenberg, G. J. Adema, and C. G. Figdor. 1994. Melanocyte lineage-specific antigen gp100 is recognized by melanoma-derived tumor-infiltrating lymphocytes. *J. Exp. Med. 179:1005.*
27. Kawakami, Y., R. Zakut, S. L. Topalian, H. Stotter, and S. A. Rosenberg. 1992. Shared human melanoma antigens: recognition by tumor-infiltrating lymphocytes in HLA-A2.1-transfected melanomas. *J. Immunol. 148:638.*
28. Bakker, A. B., S. H. van der Burg, R. J. Huijbens, J. W. Drijfhout, C. J. Melief, G. J. Adema, and C. G. Figdor. 1997. Analogues of CTL epitopes with improved MHC class-I binding capacity elicit anti-melanoma CTL recognizing the wild-type epitope. *Int. J. Cancer 70:302.*
29. Nijman, H. W., J. G. Houbiers, M. P. Vierboom, S. H. van der Burg, J. W. Drijfhout, J. D'Amaro, P. Kenemans, C. J. Melief, and W. M. Kast. 1993. Identification of peptide sequences that potentially trigger HLA-A2.1-restricted cytotoxic T lymphocytes. *Eur. J. Immunol. 23:1215.*
30. van der Burg, S. H., E. Ras, J. W. Drijfhout, W. E. Benckhuijsen, A. J. Bremers, C. J. Melief, and W. M. Kast. 1995. An HLA class I peptide-binding assay based on competition for binding to class I molecules on intact human B cells: identification of conserved HIV-1 polymerase peptides binding to HLA-A*0301. *Hum. Immunol. 44:189.*
31. Lessin, S. R., A. H. Rook, and G. Rovera. 1991. Molecular diagnosis of cutaneous T-cell lymphoma: polymerase chain reaction amplification of T-cell antigen receptor β-chain gene rearrangements. *J. Invest. Dermatol. 96:299.*
32. Lamers, C. H., R. A. Willemsen, B. A. Luider, R. Debets, and R. L. Bolhuis. 2002. Clinical protocol for gene transduction and expansion of human T lymphocytes for immunogene therapy of cancer. *Cancer Gene Ther. 7:61.*3.

33. Gratama, J. W., R. J. Bruin, C. H. Lamers, R. Oosterom, E. Braakman, G. Stoter, and R. L. Bolhuis. 1993. Activation of the immune system of cancer patients by continuous i.v. recombinant IL-2 (rIL-2) therapy is dependent on dose and schedule of rIL-2. *Clin. Exp. Immunol. 92:185.*
34. Weijtens, M. E., R. A. Willemsen, E. H. Hart, and R. L. Bolhuis. 1998. 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 Ther. 5:1195.*
35. Chothia, C., D. R. Boswell, and A. M. Lesk. 1988. The outline structure of the T-cell $\alpha\beta$ receptor. *EMBO J. 7:3745.*
36. Arden, B., S. P. Clark, D. Kabelitz, and T. W. Mak. 1995. Human T-cell receptor variable gene segment families. *Immunogenetics 42:455.*
37. Ruppert, J., J. Sidney, E. Celis, R. T. Kubo, H. M. Grey, and A. Sette. 1993. Prominent role of secondary anchor residues in peptide binding to HLA-A2.1 molecules. *Cell 74:929.*
38. Sykulev, Y., M. Joo, I. Vturina, T. J. Tsomides, and H. N. Eisen. 1996. Evidence that a single peptide-MHC complex on a target cell can elicit a cytolytic T cell response. *Immunity 4:565.*
39. Moss, P. A., and J. I. Bell. 1996. Comparative sequence analysis of the human T cell receptor TCRA and TCRB CDR3 regions. *Hum. Immunol. 48:32.*
40. Garboczi, D. N., P. Ghosh, U. Utz, Q. R. Fan, W. E. Biddison, and D. C. Wiley. 1996. Structure of the complex between human T-cell receptor, viral peptide and HLA-A2. *Nature 384:134.*
41. Visseren, M. J., S. H. van der Burg, G. E. Hawes, E. I. van der Voort, P. J. van den Elsen, and C. J. Melief. 1997. Affinity, specificity and T-cell-receptor diversity of melanoma-specific CTL generated in vitro against a single tyrosinase epitope. *Int. J. Cancer 72:1122.*
42. Romero, P., C. Pannetier, J. Herman, C. V. Jongeneel, J. C. Cerottini, and P. G. Coulie. 1995. Multiple specificities in the repertoire of a melanoma patient's cytolytic T lymphocytes directed against tumor antigen MAGE-1.A1. *J. Exp. Med. 182:1019.*
43. Dembic, Z., W. Haas, S. Weiss, J. McCubrey, H. Kiefer, H. von Boehmer, and M. Steinmetz. 1986. Transfer of specificity by murine α and β T-cell receptor genes. *Nature 320:232.*
44. Cole, D. J., D. P. Weil, J. Shilyansky, M. Custer, Y. Kawakami, S. A. Rosenberg, and M. I. Nishimura. 1995. Characterization of the functional specificity of a cloned T-cell receptor heterodimer recognizing the MART-1 melanoma antigen. *Cancer Res. 55:748.*
45. Liu, X., E. A. Peralta, J. D. Ellenhorn, and D. J. Diamond. 2000. Targeting of human p53-overexpressing tumor cells by an HLA A*0201-restricted murine T-cell receptor expressed in Jurkat T cells. *Cancer Res. 60:693.*
46. Pollok, K. E., H. Hanenberg, T. W. Noblitt, W. L. Schroeder, I. Kato, D. Emanuel, and D. A. Williams. 1998. High-efficiency gene transfer into normal and adenosine deaminase-deficient T lymphocytes is mediated by transduction on recombinant fibronectin fragments. *J. Virol. 72:4882.*
47. Chang, H. C., Z. Bao, Y. Yao, A. G. Tse, E. C. Goyarts, M. Madsen, E. Kawasaki, P. P. Brauer, J. C. Sacchettini, and S. G. Nathenson. 1994. A general method for facilitating heterodimeric pairing between two proteins: application to expression of α and β T-cell receptor extracellular segments. *Proc. Natl. Acad. Sci. USA 91:11408.*
48. Fitzer-Attas, C. J., D. G. Schindler, T. Waks, and Z. Eshhar. 1998. Harnessing Syk family tyrosine kinases as signaling domains for chimeric single chain of the variable domain receptors: optimal design for T cell activation. *J. Immunol. 160: 145.*
49. Nakagomi, H., M. Petersson, I. Magnusson, C. Juhlin, M. Matsuda, H. Mellstedt, J. L. Taupin, E. Vivier, P. Anderson, and R. Kiessling. 1993. Decreased expression of the signal-transducing ζ chains in tumor- infiltrating T-cells and NK cells of patients with colorectal carcinoma. *Cancer Res. 53:5610.*
50. Finke, J. H., A. H. Zea, J. Stanley, D. L. Longo, H. Mizoguchi, R. R. Tubbs, R. H. Wiltrout, J. J. O'Shea, S. Kudoh, and E. Klein. 1993. Loss of T-cell receptor ζ chain and $p56^{lck}$ in T-cells infiltrating human renal cell carcinoma. *Cancer Res. 53:5613.*