Chapter 6

Selecting highly affine and well-expressed TCRs for gene therapy of melanoma.


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(*these authors contributed equally to this work)
A recent phase 1 trial has demonstrated that the generation of tumor-reactive T lymphocytes by transfer of specific T-cell receptor (TCR) genes into autologous lymphocytes is feasible. However, compared with results obtained by infusion of tumor-infiltrating lymphocytes, the response rate observed in this first TCR gene therapy trial is low. One strategy that is likely to enhance the success rate of TCR gene therapy is the use of tumor-reactive TCRs with a higher capacity for tumor cell recognition. We therefore sought to develop standardized procedures for the selection of well-expressed, high-affinity, and safe human TCRs. Here we show that TCR surface expression can be improved by modification of TCR alpha and beta sequences and that such improvement has a marked effect on the in vivo function of TCR gene-modified T cells. From a melanoma-reactive panel of human TCRs we subsequently selected the TCR with the highest affinity. Furthermore, a generally applicable assay was used to assess the lack of alloreactivity of this TCR against a large series of common human leukocyte antigen alleles. The procedures described in this study should be of general value for the selection of well- and stably expressed, high-affinity, and safe human TCRs for subsequent clinical testing.

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Materials and methods

Mice
Six- to 10-week-old female C57BL/6 mice (H-2^d) and pmel-1 TCR transgenic mice were obtained from the Experimental Animal Department of the Netherlands Cancer Institute. All animal experiments were performed in accordance with institutional and national guidelines and were approved by the Experimental Animal Committee of the Netherlands Cancer Institute (DCC).

Cell lines and peripheral blood mononuclear cells
FLYR18 is a human fibrosarcoma retroviral packaging cell line (ECACC no. 95091902). The Jurkat/MA cell line is a Jurkat cell line lacking the endogenous TCR expression. The HLA-A^2 cell line that is deficient for TAP (transporter associated with antigen presentation). The panel of single major histocompatibility complex (MHC) class I-deficient Mel cells was established in the NKI from Melanoma cell lines Mel 526 (HLA-A^2, Gp100^+), Mel 938 (HLA-A^2, Gp100^+, Mart^+), and Mel1938 (HLA-A^2, Gp100^+, Mart^+). Sequences of the Gp100-specific pmel-1 TCR were kindly provided by N. Restifo (NIH, Bethesda, MD). Sequences of the Mart-1–specific ID3 and 2C2 TCRs have been described previously. The Mart-1–specific DM14 TCR and the Gp100-specific R6C12 TCR were isolated at the NIH. Modified TCR genes were designed and produced by GeneArt (Regensburg, Germany). DNA sequences are provided in Figure S2, available on the Blood website; see the Supplemental Figures link at the top of the online article. Wild-type wt and gene-optimized TCR alpha and beta chains were cloned into the retroviral vector pMX2 containing an internal ribosomal entry site, or in the indicated vector. Production of retroviral supernatants and retroviral transduction
FLYR18 packaging cells were plated in 6-well plates at 1.5 \times 10^{5} cells/well. After one day, cells were transfected with 2.5 \mu g retroviral vector DNA using FuGENE TM6 (Roche Diagnostics, Indianapolis, IN). After 48 hours, retroviral supernatant was pooled, centrifuged, and frozen at –80°C. PBMCs were activated with 20 U/mL IL-2 and 2 mg/mL phytohemagglutinin, at 1 \times 10^{6} cells/mL. Forty-eight hours after stimulation, PBMCs were resuspended in retroviral supernatant, transferred to RetroNectin-coated plates at 0.5 \times 10^{6} cells/mL, and centrifuged for 90 minutes at 430g. Jurkat/MA cells were transduced without the centrifugation step. Mouse splenocytes were transduced as described previously.

Flow cytometric analysis
Surface expression of pmel-1 TCR-transduced murine splenocytes was measured using D-terramers, or by double staining with fluorescein isothiocyanate (FITC)-labeled anti-V_{\beta}13 monoclonal antibody (mAb) and PE-labeled anti-V_{\beta}2, 3, 4, 5, 8, 9, 10, and 11 mAb (anti-V_{\beta}-pool), in combination with anti-CD8 mAb (all mAbs from BD Pharmingen, San Jose, CA). Surface expression of TCR-transduced PBMCs was measured by staining with MHC-terramers, using MHC-terramers generated through ultraviolet-peptide exchange, or by staining with anti-V_{\beta}12 (for DMF4), anti-V_{\beta}14 (for ID3 and 2C2), or anti-V_{\beta}8 (for R6C12) antibody (Immunotech, Westbrook, ME), in combination with anti-CD8 or anti-CD4 antibody (Becton Dickinson, San Jose, CA). Cells were analyzed and sorted using a FACSCalibur and FACSAria (Becton Dickinson).

Adaptive transfer and viral infection
Mice received an intravenous adaptive transfer of transduced splenocytes, transduced splenocytes, or in vitro-activated splenocytes from pmel-1 transgenic mice. To induce lymphodepletion, mice received total body irradiation (TBI) of 5 Gy, one day before adaptive transfer. Mice were vaccinated at the day of adoptive transfer by intraperitoneal injection of 1 \times 10^{7} plaque-forming units of recombinant vaccinia virus encoding hGp100_{526-542}, kindly provided by N. Restifo. For the measurement of T-cell responses, peripheral blood samples were taken at the indicated days after treatment.

IFN-\gamma assay
T2 cells were pulsed with peptides for 1 to 2 hours at 37°C. Next, 0.5 \times 10^{6} TCR-transduced PBMCs were incubated with 0.5 \times 10^{6} peptide-pulsed T2 cells or 0.5 \times 10^{6} SALS, in the presence of 20 U/mL IL-2 and 1 \mu g/mL Golgiplug (BD Biosciences, Basel, Switzerland). After 4- to 5-hour incubation at 37°C, cells were washed and stained with FITC-labeled anti-CD8 antibody and phycoerythrin (PE)-labeled anti-CD4 antibody, and analyzed for IFN-\gamma production by intracellular cytokine staining.

Chromium release assay
Target cells were labeled for 1 hour at 37°C with 100 \mu Ci (3.7 MBq) ^{51}Cr (Amersham, Gent, Belgium). Labeled target cells were incubated with effector cells at indicated ratios for 4 hours at 37°C in 200 \mu L medium, in the presence of a 50-fold excess of unlabeled K562 cells.

Results
Increased expression in and vivo function of gene-optimized TCRs
A specific issue in TCR gene transfer has been the relatively low level of transgene expression that is obtained after retroviral modification of human T lymphocytes. This is at least partially due to competition of introduced and endogenous TCR gene products for the limited pool of CD3 components, and inefficient heterodimer formation of the introduced TCR chains.

It has previously been demonstrated that in vitro TCR expression can be improved by the use of synthetic genes with an optimized codon usage. However, the in vivo consequences of TCR gene optimization—which will determine its utility in a clinical setting—have not been assessed. To address this issue, the nucleic acid sequence of the murine Gp100–specific pmel-1 TCR was modified to conform to the codon bias observed in highly expressed mammalian genes, thereby avoiding cis-acting sequence motifs.

To distinguish pmel-1 expression from endogenous TCR expression, mouse splenocytes, transduced with retroviruses containing the wt or optimized (opt) pmel-1 TCR genes, were stained in combination with a mixture of anti-V_{\beta}13 antibodies and a pool of anti-V_{\beta} antibodies, thereby allowing detection of T cells with dual TCR expression. Analysis of TCR-transduced cells via this strategy...
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(Figure 1A) or with MHC-tetramers (Figure 1B) revealed a substantial increase in the percentage of cells expressing the pmel-1 TCR upon gene optimization (Vβ) staining (44.5% versus 18.2%; MHC-tetramer staining 19.5% versus 4.7%). The level of expression was affected to a lesser extent (mean fluorescence intensity [MFI]: 63 versus 52). The markedly lower percentage of MHC-tetramer+ cells compared with the percentage of Vβ1+ cells can be explained by the fact that T cells with relatively low TCR expression can escape detection by MHC-tetramer staining. Furthermore, T cells in which the introduced TCR beta chain is expressed as a mixed heterodimer with endogenous alpha chains are detected by Vβ staining, but not MHC-tetramer staining.32,33 In addition, when single anti-Vβ staining is used (as for human cells, Figure 2A), T cells with endogenous TCRs expressing this beta chain will also be detected.

To test whether gene optimization affected the in vivo function of TCR-modified T cells, B6 mice received an adoptive transfer of WT or OPT TCR-transduced cells. As a control, mice received nontransduced cells or splenocytes of pmel-1 transgenic mice. Remarkably, although mice received an equal number of TCR-transduced cells, T-cell responses upon infection with a recombinant vaccinia strain encoding the hGp100(25-33) epitope were markedly higher in recipients of OPT pmel-1 TCRs than in recipients of WT pmel-1 TCRs, and comparable with responses in recipients of pmel-1 TCR-transgenic cells (Figure 1C, peak T-cell responses of 13% and 39% for WT and OPT, respectively).

Adoptive transfer of transduced cells to irradiated recipients, a setting resembling TCR gene transfer in lymphodepleted patients, confirmed the marked difference between WT and OPT pmel-1- transduced cells and revealed long-term persistence of cells transduced with gene-optimized TCRs (Figure 1D). Furthermore, an increased in vivo antigen-specific T-cell responses upon TCR gene optimization is likewise observed for a second TCR (OTI-L, de Witte et al, unpublished observations, December 2006).

To assess the effect of TCR gene optimization of human melanoma-specific TCRs, we generated wild-type and gene-optimized versions for 2 human TCRs (Mart-1–specific DMF4 and Gp100-specific R6C12). As for the murine TCR, gene optimization led to a substantial increase in the percentage of cells with detectable transgene expression in transduced PBMCs (Figure 2A: Vβ staining, Figure 2B: MHC-tetramer staining). Gene optimization of the DMF4 TCR resulted in a 2-fold increase in the percentage of Vβ12-expressing cells (36.8% compared with 17.0%) and an almost 3-fold increase in tetramer-binding cells (24.7% compared with 9.0%). In R6C12 TCR-transduced cells, gene modification resulted in a 3-fold increase in Vβ8-expressing cells (32.9% versus 11.3%) and a more than 7-fold increase in tetramer-binding cells (22.4% versus 3.0%). Gene optimization predominantly affected the percentage of T cells with detectable transgene expression, whereas the level of TCR expression was enhanced to a lesser extent (MFI: MHC-tetramer+ cells OPT versus WT TCR: DMF4, 127 versus 100; R6C12, 98 versus 63). This effect of gene optimization was observed using a series of independent transductions (n = 4) and independent DNA batches (n = 2).

As the effect of gene optimization is thought to be in part due to enhanced RNA stability, it was possible that the improved expression of gene-modified TCRs was partially caused by more efficient production of retroviral particles. To be able to distinguish between an effect of gene optimization on TCR protein production and on viral titers, transductions were performed in a cell line that is devoid of endogenous TCR cell surface expression. In these Jurkat/MA cells, exogenous TCR alpha and beta chains are expressed at the cell surface in the absence of competition with endogenous TCR chains.

Whereas transduction with wild-type and gene-optimized TCRs resulted in a marked difference in expression in PBMCs, transduction of Jurkat/MA cells with serial dilutions of retrovirus revealed comparable expression for gene-optimized and wild-type TCRs, both for the DMF4 (Figure 2C) and the R6C12 TCR (Figure 2D). This indicates that gene optimization has an effect primarily on TCR protein production, whereas retroviral titers are not measurably influenced.
Having established the in vitro and in vivo benefit of TCR gene optimization, we sought to select the TCR with the highest expression and functional activity from a panel of 4 gene-optimized TCRs. This panel consisted of 3 Mart-1–specific TCRs (1D3, 2C2, and DMF4) and a Gp100-specific TCR (R6C12). The 1D3 and 2C2 TCRs were derived from highly tumor-reactive cytotoxic T lymphocyte (CTL) clones isolated from a melanoma patient vaccinated with Mart-126-35 peptide. The DMF4 TCR was isolated from a dominant T-cell clone in a patient who experienced an objective tumor regression upon adoptive transfer of autologous TILs. This receptor was used in the recent TCR gene transfer trial. The R6C12 TCR was derived from a CTL clone of a melanoma patient vaccinated with Gp100 209-217 peptide.

First, we assessed the relative efficacy with which the 4 different TCRs were expressed at the cell surface. To rule out differences in retroviral titers of the TCR panel, retroviral supernatants were titrated in the Jurkat/MA cell line.

Figure 3. Differential expression of a panel of optimized melanoma-specific TCRs in peripheral blood T cells. Jurkat/MA cells were transduced with titrated aliquots of viral supernatants of vectors encoding the gene-optimized Mart-1–specific TCRs 1D3, 2C2, and DMF4 or the Gp100–specific TCR R6C12. Transduced cells were stained with anti-TCR or anti-CD8 4 days after transduction. Histograms show levels of TCR expression for the different TCRs. (B) Retroviral aliquots used in panel A were used to transduce human peripheral blood T cells. TCR expression was determined by staining with anti-CD8 and A2.1-Mart-1(26-35, 27 A/H11022 L) or A2.1-Gp100(209-217, 210 T/H11022 M) tetramers. The numbers in the upper-right and lower-right corners indicate the percentage of tetramer CD8 and tetramer CD8 cells, respectively.

Expression patterns and activity of a panel of melanoma-specific TCRs

Having established the in vitro and in vivo benefit of TCR gene optimization, we sought to select the TCR with the highest expression and functional activity from a panel of 4 gene-optimized TCRs. This panel consisted of 3 Mart-1–specific TCRs (1D3, 2C2, and DMF4) and a Gp100–specific TCR (R6C12). The 1D3 and 2C2 TCRs were derived from highly tumor-reactive cytotoxic T lymphocyte (CTL) clones isolated from a melanoma patient vaccinated with Mart-126-35 peptide. The DMF4 TCR was isolated from a dominant T-cell clone in a patient who experienced an objective tumor regression upon adoptive transfer of autologous TILs. This receptor was used in the recent TCR gene transfer trial. The R6C12 TCR was derived from a CTL clone of a melanoma patient vaccinated with Gp100209-217 peptide.

First, we assessed the relative efficacy with which the 4 different TCRs were expressed at the cell surface. To rule out differences in retroviral titers of the TCR panel, retroviral supernatants were titrated in the Jurkat/MA cell line. Virus dilutions resulting in comparable expression of the 4 different TCRs in Jurkat/MA (Figure 3A). Titration shown in Figure...
with Mart-1(26-35) peptide–loaded targets resulted in cytokine production of PBMCs, transduced with titrated virus, was determined. Incubation with 1D3 giving the highest percentage of MHC-tetramer in CD8 receptors, and induced IFN- expressed, had a higher sensitivity than the 2 other Mart-1–specific receptors, and a substantial population of MHC-tetramer staining in CD8 cells transduced with either one of the Mart-1–specific receptors (Figure 4A). However, the 1D3 receptor was approximately 10-fold more sensitive than 2C2 or DMF4 (EC50: 1D3: 3-10 nM, 2C2 and DMF4: 30-100 nM). Furthermore, modification of PBMCs with the 1D3 receptor also provided CD4+ cells with the capacity for IFN-γ production upon antigen recognition (EC50: 30-100 nM), whereas this was not observed for the 2C2 or DMF4 receptor (Figure 4B). Transduction of PBMCs with the R6C12 receptor resulted in only low levels of IFN-γ production in CD8+ cells upon incubation with Gp100(209-217)–loaded targets (Figure 4C, EC50: 1 nM), and no detectable production in CD4+ cells (Figure 4D). Of the Mart-1–specific TCRs, the 1D3 TCR was selected for further study, as it was well expressed, had a higher sensitivity than the 2 other Mart-1–specific receptors, and induced IFN-γ production in both CD8+ and CD4+ cells. Peptide titrations are not useful to compare the relative effectiveness of T-cell receptors recognizing distinct epitopes (ie, the 1D3 and 2C2 receptors) recognizing the Mart-1 and Gp100 epitope, respectively, as differences in epitope density on target cells are not taken into account. To address this issue, the cytotoxic activity of peripheral blood T cells modified with either the Mart-1–specific 1D3 or the Gp100-specific R6C12 receptor was tested against several HLA-A2+ human melanoma cell lines that express both target antigens. Transduction of human PBMCs with the 1D3 TCR endowed these cells with the capacity to lyse HLA-A2–positive melanoma cell lines, whereas HLA-A2–negative target cells were not killed (Figure 4E). In contrast, lysis by cells transduced with the R6C12 TCR was substantially lower, and only slightly above background (Figure 4F).

The use of gene optimization and subsequent in vitro comparison of different TCRs yielded a synthetic 1D3 alpha-beta gene pair that is well expressed and highly active. To formally test the value of these optimizations, PBMCs transduced with titrated virus encoding the opt 1D3 receptor were compared with PBMCs transduced with the wt DMF4 TCR that was recently used in a clinical trial. In addition, the opt DMF4 TCR was included to determine whether gene optimization affects primarily the yield of cells showing detectable transgene expression, or also T-cell sensitivity.

MHC-tetramer staining confirmed expression patterns observed in previous experiments (Figure 5A), with gene-optimized 1D3 showing the highest percentage of MHC-tetramer+ CD8+ cells (11.7%), and gene-optimized DMF4 showing a markedly higher percentage than its wild-type counterpart (9.1% vs 2.9%). Furthermore, only 1D3 showed a substantial population of MHC-tetramer+ CD4+ cells (8.8%).
Figure 5. Comparison of the gene-optimized 1D3 TCR and wild-type DMF4 TCR. Activated PBMCs were transduced with viral vectors encoding the DMF4 wild-type or gene-optimized receptor or the 1D3 gene-optimized receptor. (A) Four days after transduction, expression in PBMCs was determined by staining with anti-CD8 and A2.1-Mart1-1D3 tetramer. The numbers in the top-right and bottom-right corners indicate the percentage of tetramer+ CD8+ and tetramer+ CD8+ cells, respectively. (B) Transduced cells were incubated with T2 cells loaded with the indicated Mart-1 (26-35) peptide concentrations. After 5 hours of incubation, cells were stained with anti-CD8, and intracellular cytokine production was determined by anti-IFN-γ staining. The percentage of IFN-γ+ CD8+ cells is shown for wild-type DMF4 (●), gene-optimized DMF4 (▲), gene-optimized 1D3 (■), or nontransduced lymphocytes (○). Error bars represent standard deviations (n = 2). (C) Tetramer+ CD8+ cells were sorted and 1 week later incubated with T2 cells loaded with the indicated Mart-1 (26-35) peptide concentrations. After 5 hours of incubation, cells were stained with anti-CD8, and intracellular cytokine production was determined by anti-IFN-γ staining. The percentage of IFN-γ+ CD8+ cells is shown for sorted wild-type DMF4 (●), gene-optimized DMF4 (▲), and gene-optimized 1D3 (■) transduced PBMCs.

In line with the data shown in Figure 4A, optimized 1D3 TCR–transduced cells were approximately 10-fold more sensitive than transduced cells with the optimized DMF4 TCR. Comparison of wild-type and optimized DMF4 revealed equal sensitivity, indicating that peptide sensitivity is not measurably affected by gene optimization (Figure 5B).

The percentage of T cells transduced with the unmodified DMF4 TCR that was detected in functional assays was high compared with the percentage of transgene expression as detected by MHC-tetramer staining. This could either reflect the fact that MHC-tetramer staining underestimates the frequency of TCR-modified T cells or that T cells expressing the unmodified DMF4 are more likely to be functionally active compared with T cells expressing other Mart-1–specific TCRs. To test this, 1D3 opt, DMF4 opt, and DMF4 wt TCR-transduced cells were sorted on the basis of MHC-tetramer and CD8 staining (resulting in 84%, 80%, and 82% tetramer+ CD8+ cells, respectively; data not shown), and IFN-γ production was determined upon peptide stimulation (Figure 5C). Sorted PBMCs transduced with either wild-type or gene-optimized DMF4 receptor showed similar percentages of IFN-γ–producing cells. Furthermore, PBMCs transduced with gene-optimized 1D3 maintained their higher sensitivity after sorting.

Recognition of allogeneic MHC molecules

As discussed previously,5 TCR gene transfer in the clinical setting entails a partial MHC mismatch between the TCR recipient and the original “TCR donor.” Consequently, during thymic selection, the introduced TCR has not been selected against reactivity toward all of the MHC alleles expressed by the recipient, and recognition of allogeneic MHC molecules complexed with self-antigens could result in autoimmune pathology. Although alloreactivity has not been observed for the TCR used in a first phase 1 trial,14 the high frequency of T-cell alloreactivity in other settings21 warrants the development of standardized methods to screen for such reactivity.

To mimic a setting in which there is an MHC mismatch between TCR recipient and donor, PBMCs transduced with the 1D3 receptor were tested against a large panel of K562 cell lines each expressing single HLA-A and -B alleles (the SAL panel)21 by determining intracellular IFN-γ production upon coculture. Of the 21 SALs tested, none induced detectable IFN-γ production in 1D3-expressing CD8+ or CD4+ cells, whereas incubation with Mart-1 (26-35)–peptide–loaded T2 cells or HLA-A2+ SALs did result in a substantial population of CD8+ and CD4+ cells producing IFN-γ (Figure 6). These results show that for the receptor and MHC alleles tested, alloreactivity does not seem to play an important role.

Long-term in vitro culture and vector comparison

As long-term persistence of adoptively transferred cells is thought to be important for clinical antitumor efficacy,18 we set out to determine whether PBMCs transduced with the gene-optimized 1D3 receptor would maintain TCR expression and cytotoxic capacity after prolonged in vitro culture.

Every 3 or 4 days, total cell numbers (Figure 7A) and percentage of MHC-tetramer+ cells (Figure 7B) were assessed. A high percentage of MHC-tetramer+ cells was maintained over 4 weeks of culture, even after a 3-log expansion. Interestingly, in line with previous data on the effect of T-cell activation state on activity of the retroviral LTR,20 the fraction of MHC-tetramer+ cells was highest in the period following T-cell restimulation. After prolonged in vitro culture, 1D3 TCR–transduced cells remained capable of lysing HLA-A2+–positive melanoma cell lines (Figure 7C).

Finally, having selected a well- and stably expressed, highly affine, melanoma-specific TCR with no detectable alloreactivity against a large series of HLA alleles, we aimed to assess which retroviral vector is most suitable for expression of TCR transgenes in human lymphocytes. To this purpose, 3 different
retroviral vectors that have previously been used in clinical trials (pBullet, pMP71.90, and SFCMM5) were compared with the pMX vector used in in vivo mouse studies for their ability to yield TCR transgene expression in human PBMCs, and the same set of retroviral vectors was also evaluated in the Jurkat/MA system. The Mart 1D3 receptor was cloned into each vector in either an α-IRE-β or α-SV40EP β configuration. All vectors were capable of inducing expression of the 1D3 receptor in the Jurkat/MA system (Figure 7D). In contrast, only transduction with pMX- and pMP71.90-based vectors yielded efficient expression of the 1D3 TCR in PBMCs, resulting in 10.8% (MFI: 261) and 6.2% (MFI: 323) tetramer-binding cells, respectively. These data support 2 points, first evaluation of TCR expression in cell lines such as Jurkat/MA cells has little predictive value for the capacity of these vectors to yield detectable TCR expression in peripheral blood T cells. Second, for the expression of TCR alpha and beta genes from a single vector, pMP71.90 is the most effective of those vectors previously used in a clinical setting, and comparable with the pMX vector that has successfully been used in a series of in vivo mouse studies.

**Discussion**

Here, we describe a 3-step approach for the selection of well-expressed, high-affine, and safe TCRs. An important step in this approach is the improvement of TCR expression via gene modification, since expression of introduced TCR alpha and beta genes is generally low due to formation of heterodimers of endogenous and introduced chains, resulting in lower expression of the correctly paired heterodimer. Furthermore, correctly assembled chains compete with other heterodimers for binding to the CD3 complex that forms the limiting component in the TCR assembly process.

We modified TCR genes to improve stability and translation of the messenger RNA while leaving the amino acid sequence unaltered, thus enabling efficient protein expression. We were able to show that cell surface expression of 2 of 3 murine TCRs and 2 of 2 human TCRs benefit substantially from gene optimization (this paper and A.J., unpublished observations, September 2006).

Furthermore, gene modification of the murine pmel-1 TCR distinctly improves in vivo antigen recognition, as determined by expansion of the transduced T-cell population upon vaccination. Interestingly, this marked effect on the in vivo expansion of TCR-modified cells was observed even though the increase in the level of expression upon gene optimization was only modest. As a comparable increase in expression is observed upon gene optimization of 2 human TCRs, these data suggest that the in vivo behavior of human TCR-transduced PBMCs may also benefit substantially from gene optimization. Likewise, the current data suggest that the modest increase in expression of exogenous TCRs upon remodeling of the TCRβ interface may also result in a substantially improved engraftment of TCR-modified cells in vivo.

Notably, the effect of gene optimization was more pronounced for the human Gp100-specific R6C12 receptor than for the Mart-1-specific DMF4 receptor, both with respect to the percentage of cells showing detectable transgene expression and the level of expression. To assess whether it may be possible to predict the value of gene optimization for different TCRs, we determined the percentage of codons that was modified in the variable regions of these receptors. In the gene-optimized R6C12 receptor, 52% of codons were modified in the alpha chain and 47% in the beta chain. In contrast, modifications of DMF4 alpha and beta chains were 32% and 21%, respectively. This may suggest that for TCRs for which a lower level of gene optimization is required, this process results in a smaller increase in TCR expression. However, a substantially larger dataset will be required to rigorously test this notion.

In addition to demonstrating the in vivo effect of TCR gene optimization, the data described in this study show that selection of both viral platforms and individual TCRs should occur by analysis of expression in peripheral blood T cells rather than cell systems that lack endogenous TCR expression, in which the requirements for expression of exogenous TCRs are substantially lower. As an example, while expression of R6C12 and 1D3
receptors is comparable in Jurkat/MA cells, only the latter is efficiently expressed in human PBMCs. Likewise, while all 4 retroviral vector systems tested yielded substantial TCR expression in Jurkat/MA cells, pMX and pMP71 were superior in yielding TCR expression in PBMCs.

Clinical application of TCR gene transfer should be preceded by an evaluation of the possible side effects, caused by either on-target reactivity (mediated via recognition of target antigens on normal tissues) or off-target reactivity (mediated by mechanisms described in the next paragraph). The risk of on-target autoimmunity will primarily depend on the expression pattern of the antigen involved. Melanoma-differentiation antigens, such as Mart-1 and Gp100, are expressed on melanoma cells as well as normal melanocytes, and targeting of these antigens is known to induce autoimmune melanocyte destruction resulting in vitiligo and uveitis.\textsuperscript{5,26} TCR gene transfer targeting these antigens has not resulted in severe side effects.\textsuperscript{9} However, it remains possible that increased autoreactivity will occur when more potent TCRs or conditioning regimens are used.

Off-target autoimmunity by TCR-transduced cells theoretically can be induced via 3 different mechanisms.\textsuperscript{5} First, introduction of exogenous TCR chains can lead to the formation of heterodimers with endogenous alpha and beta chains, which might be reactive toward self-peptides. Second, if ignorant self-reactive T cells are transduced, triggering of these cells via the introduced TCR can result in an expanded population of autoreactive cells. Off-target autoreactivity via these 2 mechanisms would occur irrespective of MHC disparities between TCR donor and recipient. Both mouse models of TCR gene transfer\textsuperscript{12,15} and the recent phase 1 clinical trial\textsuperscript{41} do not provide evidence that these mechanisms form a substantial reason for concern.

As a third possibility for off-target autoimmunity, MHC mismatches between TCR donor and recipient may result in recognition of allelic MHC molecules complexed to self-antigens by the TCR-modified cells. With the aim to develop a generally applicable strategy to screen for such autoreactivity, we developed a simple assay to test TCR-modified T cells against a set of cell lines expressing defined single HLA-A and -B alleles with a high prevalence in the human population. Lack of reactivity against any of the MHC alleles expressed by this panel provides evidence that for this T-cell receptor the risk of type III off-target autoimmunity may be little. However, it may be worthwhile to further expand the set of class I alleles that is tested for a more complete evaluation of MHC autoactivity, and possibly such an evaluation should also include reactivity against MHC class II alleles. Furthermore, although allorecognition by TCRs is often less peptide dependent,\textsuperscript{46} it remains possible that a TCR that is unreactive toward an MHC allele on the cell line used for in vitro testing does recognize this MHC allele when complexed with a tissue-specific antigen. Based on this latter consideration, it may remain useful to set up a database for allowed MHC mismatches for every TCR used in coming clinical trials.

We have used this evaluation and optimization strategy to select a melanoma-specific receptor with improved expression, a higher affinity compared with other receptors, and a lack of detectable alloreactivity. CD4\textsuperscript{+} T cells transduced with this MHC class I-restricted CD8 T receptor are capable of MHC-tetramer binding and production of IFN-γ. Prior data have shown that the provision of CD4\textsuperscript{+} T-cell help contributes to both primary CD8\textsuperscript{+} responses and CD8\textsuperscript{+} T-cell memory formation.\textsuperscript{9} Furthermore, recent studies in mouse models have shown that transfer of a CD8\textsuperscript{+}-dependent MHC class I-restricted TCR into CD4\textsuperscript{+} T cells can be used to generate MHC class I-restricted CD4\textsuperscript{+} T-cell help.\textsuperscript{11,12} However, as optimal function of the modified CD4\textsuperscript{+} T cells requires the presence of the CD8αβ coreceptor,\textsuperscript{13} the selection of TCRs that can function independent of CD8 is considered attractive.\textsuperscript{14,44} Perhaps more importantly, compared with a TCR evaluated in a previous clinical trial, CD8\textsuperscript{+} T cells expressing this TCR recognize antigen at a 10-fold lower concentration. Furthermore, the high level of expression of the ID3 TCR may predict a more effective in vivo persistence as based on analogy with the murine melanoma-specific pMel TCR.

The 3-step approach we describe in this study may be of use for the selection of well-expressed, high-affine, and safe T-cell receptors for future clinical trials, thereby enhancing the clinical development of TCR gene therapy. Such selection may involve the evaluation either of naturally occurring TCRs or—by analogy with antibody development—of TCRs obtained by technologies that circumvent the limitations of the naturally occurring immune repertoire.\textsuperscript{14,45,50}

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Authorship

Contribution: A.J. designed and performed research, analyzed data, and wrote the paper; R.G.-E. designed and performed research and analyzed data; M.D. performed research and analyzed data; W.K., Y.M.Z., I.I.N.D., N.R., P.R., and R.A.M. provided important reagents; T.N.M.S. and J.B.A.G.H. designed research and wrote the paper. A.J. and R.G.-E. contributed equally to this work.

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Correspondence: John B. A. G. Haanen, Division of Immunology, the Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, the Netherlands; e-mail: j.haanen@nki.nl.

References


Selecting TCRs for gene therapy of melanoma


