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T-Cell Receptor Gene Therapy of Established Tumors in a Murine Melanoma Model

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Summary

Adoptive cell transfer therapy using tumor-infiltrating lymphocytes for patients with metastatic melanoma has demonstrated significant objective response rates. One major limitation of these current therapies is the frequent inability to isolate tumor-reactive lymphocytes for treatment. Genetic engineering of peripheral blood lymphocytes with retroviral vectors encoding tumor antigen-specific T-cell receptors (TCRs) bypasses this restriction. To evaluate the efficacy of TCR gene therapy, a murine treatment model was developed. A retroviral vector was constructed encoding the pmel-1 TCR genes targeting the B16 melanoma antigen, gp100. Transduction of C57BL/6 lymphocytes resulted in efficient pmel-1 TCR expression. Lymphocytes transduced with this retrovirus specifically recognized gp100-pulsed target cells as measured by interferon- γ secretion assays. Upon transfer into B16 tumor-bearing mice, the genetically engineered lymphocytes significantly slowed tumor development. The effectiveness of tumor treatment was directly correlated with the number of TCR-engineered T cells administered. These results demonstrated that TCR gene therapy targeting a native tumor antigen significantly delayed the growth of established tumors. When C57BL/6 lymphocytes were added to antigen-reactive pmel-1 T cells, a reduction in the ability of pmel-1 T cell to treat B16 melanomas was seen, suggesting that untransduced cells may be deleterious to TCR gene therapy. This model may be a powerful tool for evaluating future TCR gene transfer-based strategies.

Keywords

murine; gene therapy; T-cell receptor; gp100 tumor antigen

Cellular adoptive immunotherapy has been shown to mediate the regression of large solid tumors in 50% of patients with metastatic melanoma.^{1,2} A limitation of this treatment is the need to isolate and expand tumor-reactive lymphocytes that preexist in the patient. Those requirements can be overcome by transduction of genes encoding antitumor T-cell receptor (TCR), thus providing an alternative source of tumor-specific lymphocytes.^{3–7} We recently reported that this approach led to objective cancer regression in 2 patients with metastatic melanoma, a response rate of 13%.⁸

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The pmel-1 mouse model was developed as a system to model treatment of malignant melanoma using adoptive cell therapy (ACT).⁹ The target antigen, *pmel-17*, is an ortholog of the melanocyte differentiation antigen gp100, which is often overexpressed in human melanomas. Adoptive transfer of transgenic T cells expressing the anti-gp100 TCR from pmel-1 mice can effectively mediate the regression of large established tumors when administered in combination with a lymphodepleting pretreatment regimen, γ_c cytokine administration, and vaccination.^{9–12} In this study, we further adapted the pmel-1 system to model TCR gene transfer therapy by retroviral vector-mediated gene transfer of the pmel-1 TCR genes into normal lymphocytes and the use of these genetically engineered cells to treat established B16 melanomas.

MATERIALS AND METHODS

Mice and Tumor Lines

C57BL/6 and pmel-1 transgenic mice (Jackson Laboratory, Bar Harbor, ME) were housed at the National Institutes of Health (NIH). The Phoenix Eco (ATCC, Rockville, MD) retroviral vector packaging line and the methylcholanthrene-induced sarcoma MCA-205 cell line (National Cancer Institute Tumor Repository, Bethesda, MD) were maintained in Iscove's 5% fetal calf serum (PCS) and RPMI 10% PCS, respectively. B16 (H-2^b), a spontaneous gp100+ murine melanoma, was maintained in RPMI 10% PCS.

Retrovirus Production

The pmel-1 TCR- α and TCR- β chain cDNA genes were inserted into vector pMX¹³ where the expression was linked by an IRES sequence to yield pMX-pmel TCR. Phoenix Eco cells/well (1.5×10^5) were placed in 6 well plates with 2-mL/well Iscove's 5% PCS. FuGENE 6 transfection reagent (6.25 μ L) (Roche, Indianapolis, IN) was added to 200 μ L of serum-free Iscove's medium for each well and incubated for 5 minutes at room temperature. In all, 2.5 μ g of pMX-pmel TCR retroviral vector or pMSGIN (GFP transgene) and 1.75 μ g of pCL-Eco (Imgenex, San Diego, CA) were added to the FuGENE for each well, incubated for 15 minutes at room temperature and added to the Phoenix Eco cells in culture. The following day, the cell medium was removed and the transfected Phoenix Eco cells were incubated overnight in fresh prewarmed Iscove's 5% FCS. The following day, the retroviral supernatant was collected from the transfected Phoenix Eco cells, spun for 10 minutes at 1000g and the supernatant removed. Recombinant human interleukin-2 (rhIL-2, Chiron, Emeryville, CA) of 60 IU/mL was added to the viral supernatant before transduction.

Retroviral Transduction

C57BL/6 spleens were harvested, macerated over a filter, and resuspended in ACK Lysing Buffer (Biosource, Rockville, MD). In all, 3×10^6 splenocytes per milliliter were placed in RPMI 10% with 1-ng/mL IL-7 and 2- μ g/mL Concanavalin A (Calbiochem, La Jolla, CA), and incubated at 37°C. Two days later, the splenocytes were collected and where indicated, CD8⁺ cells were enriched using mouse CD8 Negative Isolation Kit (DynaL Biotech, Oslo, Norway). Splenocytes were spun for 7 minutes at 486g, resuspended in retroviral supernatant with rhIL-2 at 1×10^6 cells/mL, plated on RetroNectin-coated plates (Takara Bio, Otsu, Japan), and spun at 1000g for 90 minutes at room temperature. The transduced cells were incubated at 37°C overnight. The second transductions were performed the following day using the same method. Transduced cells were in vitro stimulated with 1 μ M of hgp100_{25–33}-pulsed, irradiated C57BL/6 splenocytes 1 to 2 days after the final transduction, cultured as described,^{9,14} and used in adoptive cell transfer experiments 1 to 3 days later.

Adoptive Cell Transfer

C57BL/6 mice at 6 to 12 weeks of age were injected with 2×10^5 to 5×10^5 B16 melanoma cells and sublethally irradiated with 500 cGy 7 to 8 days later. The following day, groups of mice ($n = 5$) underwent tail vein injections of transduced C57BL/6 or pmel-1 splenocytes. Mice were vaccinated on the day of transfer with 2×10^7 PFU of recombinant fowlpox virus expressing human gp100 (rFPhgp100; Therion Biologics, Cambridge, MA) and 100,000 IU rhIL-2 administered intraperitoneally twice daily for 3 days. The perpendicular diameters of the tumors were subsequently measured with a caliper by a blinded investigator. The NCI Animal Ethics Committee of the NIH approved all animal experiments.

Flow Cytometry and TCR Activity Assay

All antibodies were purchased from BD Bioscience (San Jose, CA). The H2-D^b-mgp100₂₅₋₃₃ tetramer was purchased from Beckman Coulter (Fullerton, CA). FACSCalibur flow cytometer and CellQuest software were used to analyze the samples. Cytokine release assays and cytolytic assays were performed as previously described.¹⁵

RESULTS

Retroviral Vector Transduction of pmel-1 TCR

We previously described a murine model of human ACT based on the pmel-1 mouse.⁹ A bicistronic retroviral vector was constructed, which expressed the α and β chains of the anti-gp100 TCR expressed in pmel-1 T cells. The pmel-1 TCR retroviral vector was produced by transient transfection of an ecotropic packaging cell line and fresh supernatant was used to transduce normal T cells. One million fresh C57/B6 splenocytes were cultured for 2 days in ConA and IL-7 and then transduced with retrovirus encoding the pmel-1 TCR in the presence or absence of IL-2. At 24 hours posttransduction, both transduced cultures were analyzed by flow cytometry after staining for the pmel-1 TCR chain V β 13. A representative example of the flow cytometry is shown in Figure 1A. The C57/B6 splenocyte population transduced in the absence of IL-2 contracted approximately 20% in cell number during the transduction and approximately 12% (as measured by V β 13 staining) of the cells expressed the transgene. In comparison, C57/B6 splenocytes transduced in the presence of IL-2 demonstrated an approximate mean expansion of 1.8-fold and 29% of the cells expressed V β 13 at 24 hours posttransduction. The increased cell proliferation in IL-2 containing cultures likely accounted for the higher transduction efficiency as retroviral vectors can only productively transduce actively dividing cells. From this point, all transductions were performed with the addition of IL-2 to the retroviral supernatant.

Stable gene transfer efficiency of $> 25\%$ was consistently observed (with endogenous background staining of 1% to 5%) up to 3 days posttransduction (representative FACS shown in Fig. 1B). Two days posttransduction, cells were stimulated with gp100-specific peptide in an attempt to induce TCR-dependent proliferation. Three days poststimulation, cells were analyzed for V β 13 expression by FACS. As shown in Figure 1C, peptide stimulation resulted in a little increase in the number of V β 13+ cells (from 32% to 39%).

To increase the percentage of vector-transduced lymphocytes, we evaluated multiple transductions of CD8⁺ T-cells (Fig. 1D). C57/B6 splenocytes were placed in medium with ConA and IL-7 for 2 days, and the CD8⁺ population of splenocytes was negatively selected before transduction with the pmel-1 TCR retrovirus. At 24 hours posttransduction, the transduced CD8⁺ splenocytes were transduced a second time. Enrichment for CD8⁺ T cells before transduction increased the efficiency of gene transfer, yielding $> 50\%$ transduction as measured by V β 13+ staining (Fig. 1D). The levels of V β 13 + transgene expression and gp100 tetramer binding were only modestly increased with 2 transductions (Fig. 1D).

The biologic activity of the TCR-transduced C57/B6 cells was measured by IFN- γ secretion assay. C57/B6-transduced cell populations were cocultured with MCA-205 cells pulsed with serially diluted amounts of rhgp100 peptide (Fig. 2). After 24 hours, the supernatant was analyzed for the presence of IFN- γ by ELISA. C57/B6 splenocytes transduced with the pmel-1 TCR released IFN- γ in response to concentrations of gp100 peptide down to 1 nM, but were significantly less active than native pmel-1 T cells. The phenotypes of the C57/B6-transduced cells were analyzed for the presence of several cell surface markers associated with T-cell function: CD25, CD44, CD62L, and CD69 (data not shown). Transduction resulted in an increased level of the activation/effector marker CD25, whereas CD44 and CD69 did not demonstrate any observed change in expression after transduction or selection. The lymphoid-homing molecule, CD62L, was slightly down-regulated after transduction and ex vivo growth in IL-2. Overall, the transduction procedure resulted in cells with an intermediate effector phenotype.¹⁵

In Vivo Tumor Treatment

To test the in vivo efficacy of pmel-1 TCR-transduced PBL, we scaled up the gene transfer procedure to obtain large numbers of cells for multiple animal treatments. Seven to eight days before treatment, C57BL/6 mice were injected with 2×10^5 to 5×10^5 B16 melanoma cells and animals with well-established tumors were used for experimentation. The total number of transduced T cells administered was calculated and 1×10^6 or 7×10^6 gp100 tetramer + cells were transferred to 5 Gy irradiated, B16 tumor bearing C57/B6 mice with concurrent fowlpox virus expressing hgp100 vaccination and twice daily IL-2 administration for 3 days. Five days posttransfer, the peripheral blood of mice treated with pmel-1 TCR-transduced T cells was analyzed by flow cytometry. A representative example of the FACS data is shown (Fig. 3). The animals treated with higher amounts of transduced cells (7×10^6) had a larger percentage of V β 13 + CD8⁺ cells in the peripheral blood than mice treated with 1×10^6 cells (63% vs. 46%), but this was less than that in mice that received 1×10^6 native pmel-1 cells (81% V β 13 + CD8⁺).

The tumor size following the transfer of pmel-1 TCR-transduced cells was measured 3 times weekly to evaluate response to treatment. The group of mice treated with 7×10^6 TCR-transduced CD8⁺ splenocytes demonstrated a statistically significant delay in tumor growth compared to those treated with the same number of GFP vector-transduced cells ($P = 0.009$) (Fig. 4A). Mice treated with 7×10^6 TCR-transduced cells achieved a greater delay in tumor growth compared to those treated with 1×10^6 TCR-transduced cells ($P = 0.047$). Similar results were obtained when the number of administered cells was calculated using V β 13+ cells (Fig. 4B). Consistent delay in tumor growth was observed in 3 additional experiments where the infusion of higher numbers of cells (7×10^6 per animal) uniformly yielded a greater delay in tumor growth (data not shown).

After transduction, C57BL/6 lymphocytes contained a considerable percentage of untransduced cells, which could potentially inhibit the in vivo tumor treatment capacity. To investigate the inhibitory effect of untransduced cells, a cell mixing experiment using transgenic pmel-1 cells and normal lymphocytes was performed. Untransduced C57BL/6 CD8⁺ splenocytes were added to pmel-1 transgenic splenocytes to adjust for the total number of cells that were present when 1×10^6 (or 4×10^5) V β 13 + CD8⁺-transduced cells were transferred (eg, 1×10^6 transgenic pmel-1 T-cells plus 2.5×10^6 C57BL/6 splenocytes were administered to mimic 1×10^6 V β 13 + CD8⁺-transduced cells). The addition of transduced C57BL/6 CD8⁺ splenocytes to pmel-1 transgenic splenocytes resulted in a decrease in tumor response compared to pmel-1 transgenic cells without added splenocytes and was statistically significant (for 1×10^6 pmel-1, $P = 0.009$ and for 4×10^5 , $P = 0.014$) (Fig. 4B).

On the basis of these observations, the positive selection of V β 13 + CD8⁺ or gp100 tetramer positive cells might help to further increase the tumor treatment capacity. However, in our preliminary experiments we could not recover sufficient numbers of transduced CD8⁺ T cells after positive selection by V β 13 or gp100 tetramer binding to perform animal experiments (usually performed using 5 animals per group). The reason for the low recovery might be the relative low expression of the retroviral transduced TCR compared to the endogenous pmel-1 TCR and/or the mispairing of the introduced TCR chains with the endogenous TCR chains.

DISCUSSION

In this paper, we demonstrated that retroviral transduction of murine splenocytes lead to the expression of the pmel-1 TCR receptor that recognized the native gp100 tumor antigen. Under optimal conditions, > 50% of normal mouse CD8⁺ T cells can be engineered to express the pmel-1 TCR (as measured by V β 13 staining). When analyzed in parallel (eg, Fig. 1D), native pmel-1 T cells displayed 98% V β 13+ cells and 75% of these bound tetramer, an indicator of specific TCR α and β chain pairing. In contrast, the transduced cells demonstrated a lower fraction of tetramer binding relative to V β 13 + cells (less than 50% of V β 13 + cells). The reduced fraction of tetramer positive cells in the transduced cell population was likely the result of random pairing between the introduced pmel-1 TCR and endogenous TCR subunits. This “mispairing” may lessen the overall biologic activity of engineered T cells as observed in Figure 2, where engineered cells released significantly less IFN- γ following coculture with peptide-pulsed target cells compared to the transgenic pmel-1 T cells. It is possible that mispairing may in part be overcome by appropriate protein engineering including codon optimization and manipulation of TCR constant regions to enhance specific chain pairing.^{16–20} Recently, the concept of “strong” or “dominant” TCRs has been proposed²¹ and thus, the choice of a naturally occurring “dominant” TCR may be a effective solution to TCR chain mispairing.

Significant dose-dependent retardation in B16 tumor growth was observed in adoptive cell transfer experiments using TCR-engineered cells. Considerable improvements in tumor treatment remain to be made in this gene therapy model and could include TCR protein engineering to increase affinity^{22,23} or chain pairing,^{17,19,20} increasing the amount and duration of vector expression,²⁴ as well as, the potential use of hematopoietic stem cells to augment antitumor treatments.²⁵ In addition to the molecular aspects of optimizing the gene transfer system used herein, the biology of ACT can also be manipulated to enhance antitumor treatment.²⁶ The requirement for the stimulation of T cell growth by ConA and IL-7 is correlated with a change in cell surface markers similar to the natural shift in phenotype from a naive to a T cell with intermediate effector phenotype. Cells with this phenotype, although capable of mediating tumor regression, were demonstrated to be less active in mediating tumor regression than naive or early effector phenotype cells.^{15,26} It is possible that the use of lentiviral vectors may afford the ability to engineer minimally stimulated T cells,²⁷ which retain more of a naive or early effector T-cell phenotype. Alternatively, TCR genes can be transferred into hematopoietic stem cells and these cells differentiated into naive T cells in vitro or in vivo.^{28,29}

An unexpected observation in these studies was the significant reduction of tumor treatment when nonspecific bystander cells were added to the pmel-1 treatment cells (Fig. 4B). The transfer of antigen-specific T cells into a lymphopenic host results in homeostatic proliferation, and this can be inhibited by the cotransfer of an irrelevant population of T cells. This inhibition by bystander cells may reflect cytokine competition, the presence of inhibitory regulatory cells in the untransduced cell population, or the competition for antigen presenting cells.^{11,30,31} Enrichment for transduced cells before adoptive cell transfer was attempted with the current pmel-1 TCR vector but proved ineffective in yielding sufficient cell numbers. Our inability to purify TCR-engineered cells was possibly related to the lower level of protein expression of

the transduced α and β chain genes. A lower level of β chain expression was clearly observed in comparison to the native pmel-1 TCR as presented in Figure 3B, where the mean fluorescence intensity of V β 13 was less than half of the endogenous V β 13 protein. Further refinement in vector design to increase TCR expression or the addition of a coexpressed selectable marker gene may address this issue.

Finally, these observations extend and confirm our clinical experience demonstrating that antitumor antigen TCR gene transfer into mature lymphocytes when administered in combination with lymphodepletion, cytokine therapy, and vaccination can lead to the regression of large existing tumors.⁸ This murine adoptive immunotherapy model could be used in the future to further investigate and optimize in vivo the use of TCR encoding retroviruses and further reinforces the application of this approach for the treatment of cancer patients.

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FIGURE 1.

TCR transduction of mouse splenocytes. A, Murine C57BL/6 splenocytes were left untransduced (UT), transduced with retrovirus encoding the pmel-1 TCR in the presence or absence of IL-2 and 24 hours later were analyzed by flow cytometry for TCR Vβ13 staining. B, Following TCR transduction, samples of in vitro IL-2 cultured splenocytes were analyzed on 3 consecutive days for TCR gene expression (measured by Vβ13 staining). C, Transduced cells were stimulated with gp100-specific peptide 2 days posttransduction, and analyzed 3 days poststimulation for Vβ13 expression by FACS. D, After CD8⁺ enrichment through negative selection murine splenocytes were transduced with pmel-1 TCR vector (TD). Twenty-four hours later, a second retroviral TCR transduction was performed (TD × 2). The levels of

V β 13+ transgene expression and gp100 tetramer binding in comparison to pmel-1 T cells was evaluated by flow cytometry. Data shown are representative of 3 independent experiments.

FIGURE 2.

Function of transduced splenocytes. C57BL/6 splenocytes were transduced 1 time (TD) and co-cultured with hgp100₂₅₋₃₃-pulsed MCA-205 cells for 24 hours. The amount of interferon-γ release was analyzed by ELISA. Untransduced (NT), and transgenic pmel-1 T cells (pmel) were used as negative and positive controls, respectively.

FIGURE 3.

Persistence of TCR-transduced splenocytes after in vivo transfer. A, (In vitro) Murine C57BL/6 splenocytes were left untransduced (UT), or transduced (TD) with retrovirus encoding the pmel-1 TCR and 24 hours later analyzed by flow cytometry for TCR Vβ13 staining. B, (In vivo) The total number of CD8⁺gp100 tetramer+ transduced cells was calculated and 1×10^6 or 7×10^6 CD8⁺ gp 100 tetramer+ cells were transferred into 5 Gy irradiated, B16 tumor bearing C57BL/6 mice with concurrent fowlpox virus expressing hgp 100 vaccination and twice daily IL-2 administration for 3 days. Five days posttransfer, the peripheral blood of mice treated with pmel-1 TCR-transduced splenocytes was analyzed by flow cytometry. The mean fluorescence intensity of Vβ13 expression was as indicated in parentheses. A representative example of the FACS data is shown.

FIGURE 4.

Delayed tumor growth following transfer of TCR-transduced splenocytes. A, C57BL/6 splenocytes were transduced and 1×10^6 or 7×10^6 CD8⁺ gp100 tetramer+ cells were transferred to B16 tumor bearing C57BL/6 mice. Animals received 5 Gy TBI with concurrent fowlpox virus expressing hgp100 vaccination and twice daily IL-2 administration for 3 days. As controls, animals were either left untreated (NT) or received GFP vector-transduced (GFP-transduced) splenocytes or transgenic pmel-1 T cells (pmel-1). Results of tumor area were the mean of measurement of 5 mice per group. The delay in tumor growth in mice that received 7×10^6 CD8⁺ gp100 tetramer+ cells versus the same number of GFP vector-transduced cells was statistically significant ($P=0.009$). B, A cell mixing experiment was performed to mimic the administration of 1×10^6 CD8⁺ Vβ13+-transduced cells. Using the same percentage of untransduced cells determined in the TCR-transduced population, untransduced C57BL/6 lymphocytes were added to 1×10^6 or 4×10^5 pmel-1 transgenic splenocytes (pmel-1+UT), and compared to pmel-1 without added untransduced cells (pmel-1). After 5 Gy TBI B16 tumor bearing C57BL/6 mice received cell transfer followed by fowlpox virus expressing hgp100 vaccination and twice daily IL-2 administration for 3 days. The addition of untransduced cells to pmel-1 splenocytes had a significant negative effect on tumor treatment (1×10^6 pmel-1, $P=0.009$ and with 4×10^5 pmel-1, $P=0.014$). Tumor area is presented as the mean of 5 mice per group.