Published in final edited form as:

J Immunother. 2010 ; 33(6): 648–658. doi:10.1097/CJI.0b013e3181e311cb.

A Simplified Method for the Clinical-scale Generation of Central Memory-like CD8⁺ T Cells After Transduction With Lentiviral Vectors Encoding Antitumor Antigen T-cell Receptors

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Summary:

Adoptive transfer of antigen-specific CD8⁺ T cells can effectively treat patients with metastatic melanoma. Recent efforts have emphasized the in vitro generation of antitumor T cells by transduction of genes encoding antitumor T-cell receptors. At present, lentiviral vector-mediated transduction of CD8⁺ T cells relies on anti-CD3/CD28 bead stimulation; however, this method fails to efficiently expand CD8⁺ T cells. Herein we sought to establish a methodology for lentiviral vector transduction using optimal activating agents for efficient gene delivery and robust expansion of CD8⁺ T cells. To overcome the inability of anti-CD3/CD28 beads to efficiently expand CD8⁺ T cells, we evaluated alternative activating agents including feeder cells from allogeneic peripheral blood mononuclear cells and plate-bound anti-CD3 antibody. Analyses of gene transfer, cell phenotype, fold expansion, and biologic activities were used to determine the optimal methodology. Plate-bound anti-CD3 provided an ideal activation platform that afforded optimal lentiviral vector-mediated gene transfer efficiency (up to 90%), and coupled with peripheral blood mononuclear cells feeder cells yielded up to 600-fold expansion of CD8⁺ T cells within 12 days. The T-cell antigen receptor (TCR) engineered CD8⁺ T cells conferred specific antitumor activity and many displayed a central memory-like phenotype. The methodology described here could be readily applied for engineering CD8⁺ T cells with antitumor specificity for human adoptive immunotherapy.

Keywords

T-cell receptor; adoptive immunotherapy; CD8; central memory cells; lentivirus

Adoptive cell transfer using tumor reactive T lymphocytes is the most effective immunotherapy for patients with metastatic melanoma, reaching a 72% objective response rate.^{1,2} However, the requirement for identifying preexisting tumor responsive cells and the need for largescale ex vivo expansion limits its broad application. The genetic modification

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The authors have declared there are no financial conflicts of interest in regards to this work.

The authors declare that they have no competing financial interests.

Supplemental digital content is available for this article. Direct

of peripheral blood lymphocytes with antitumor T-cell antigen receptors (TCRs) using γ -retro-viruses readily renders autologous peripheral blood lymphocytes from any patient into tumor killing T lymphocytes in vitro. For TCR gene therapy, current protocols require T cells to be actively dividing to allow efficient γ -retroviral vector-mediated gene transfer. The use of fully activated T cells limits the number of cells capable of being transduced, and necessitates a second rapid expansion (REP) to generate enough cells for clinical applications. However, this second expansion caused cells to become fully differentiated and exhibit an effector memory phenotype that may impede in vivo persistence³ and in vivo tumor killing efficacy.⁴

Central memory CD8⁺ T cells and effector memory CD8⁺ T cells have been identified in humans and animals, and can be characterized in part by the level of CCR7 and CD62L.⁵ Klebanoff et al⁶ using a murine model concluded that central memory tumor reactive CD8⁺ T cells conferred superior antitumor reactivity compared with effector memory CD8⁺ T cells leading to the eradication of large established tumors. Recently, Berger et al³ using a macaque model showed that CD8⁺ T cells isolated from central memory T cells are distinct from those derived from effector memory T cells and retain an intrinsic capacity enabling them to survive longer after adoptive cell transfer. Most recently, Johnson et al⁷ reported that treatment of metastatic melanoma patients using modest numbers of central memory-like T cells genetically engineered to express an antitumor MART-1 TCR receptor were equally effective in mediating tumor regression than 10-fold more effector memory-like T cells.

Compared with γ -retroviral vectors, lentiviral vectors efficiently transduce nondividing cells. The minimal requirement for lentiviral vector transduction of quiescent T cells is that T cells enter into the G1 phase of the cell cycle. After anti-CD3 activation, quiescent T cells easily move into the G1 phase within hours.^{8,9} It has been reported that anti-CD3/CD28 beads could provide suitable activation for lentiviral vector-mediated transgene expression,^{8,10} but this approach favored expansion of CD4⁺ T cells^{11,12} that may compromise in vivo antitumor efficacy.^{13,14} Inefficient expansion of CD8⁺ T cells by anti-CD3/CD28 beads was also observed.¹⁵ We thus sought an alternative method for CD8⁺ T-cell stimulation.

Irradiated feeder cells from peripheral blood mononuclear cells (PBMC) plus anti-CD3 antibody have been shown to be an effective approach to expand tumor or virus-specific T-cell clones,¹⁶ tumor-infiltrating T lymphocytes (TIL) from melanoma tissues,¹⁷ and PBMC from patients with advanced metastatic melanoma.¹⁸ In this study, we compared methodologies that combined lentiviral vector transduction of CD8⁺ T cells using anti-CD3/CD28 beads, plate-bound anti-CD3 antibody, and feeder cells from allogeneic PBMC for activation and expansion. In general, CD8⁺ T cells selected by clinically approved techniques could be efficiently transduced (up to 90%). These TCR-modified CD8⁺ T cells expanded up to 600-fold within 12 days and displayed a central memory-like phenotype with pronounced in vitro antitumor activities.

MATERIALS AND METHODS

Cell Culture

PBMC used in this study were obtained from metastatic melanoma patients at the Surgery Branch, National Cancer Institute. In brief, PBMC were collected by leukapheresis, and lymphocytes were separated by Ficoll/Hypaque cushion centrifugation washed in Hank buffered salt solution and resuspended at a concentration of $1 \times 10^6/\text{mL}$ in AIM-V medium (Invitrogen, Carlsbad, CA) supplemented with 300 IU/mL IL-2 and 5% heat-inactivated human AB serum (Valley Biomedical, Winchester, VA). Culture of melanoma lines and 293 T cells was described earlier.⁸

Negative-selection and Positive-selection of CD8+ T Cells

Using the CD8+ isolation kit II (Miltenyi Biotec, Gumbrecht, Germany), CD8+ T cells were isolated from PBMC described by the supplier with a cocktail antibody to deplete non-CD8+ T cells. For positive selection of CD8+ T cells, CD8+ Microbeads (Miltenyi Biotec) were used. The detailed instructions in the kit were followed. Clinical-grade CD8+ T cells were obtained using the CliniMACS separation system with clinical-grade CD8+ Microbeads under an approved Investigational New Drug (IND) application.

Vector Construction

The lentiviral constructs used were derived from pRRLSIN.cPPT.MSCV/GFP.wPRE harboring a green fluorescent protein (GFP) gene driven by the murine stem cell virus (MSCV) U3 promoter.¹⁹ Woodchuck hepatitis response element (wPRE) was replaced with the truncated form oPRE where residual X protein and its promoter was depleted,²⁰ the oPRE sequence was synthesized fused with Sal I and EcoR I restrictive enzyme sites (Epoch Biolabs, Missouri City, TX) and cloned into corresponding sites of pRRLSIN.cPPT.MSCV/GFP.wPRE replacing wPRE, the vector named pLLV.GFP.oPRE. A lentiviral vector expressing the gp100 TCR α and β chains of DMF5 TCR targeting melanoma antigen MART-1²¹ was described earlier.^{8,22} The α and β chains of DMF5 TCR were linked with furin cleavage site (RAKR) followed by a spacer SGSG and F2A peptide; the consolidated sequence was codon-optimized and engineered with Asc I and Sal I sites by GeneArt (Regensburg, Germany), and cloned into corresponding sites of vector pRRLSIN.cPPT.MSCV/GFP.oPRE, named pLVV.coDMF5.oPRE. All the constructs are confirmed by restrictive enzyme digestion and sequencing.

Lentivirus Preparation

The day before transfection, 20×10^6 293 T cells were plated onto 150-mm² poly-D-Lysine coated plates (BD Biosciences, San Jose, CA) using 15 mL of culture medium. On the day of transfection, the medium was replaced with 15 mL fresh medium 3 hours before transfection. Each plate received plasmid DNA 55 μg (transfer vector 22.5 μg , VSV-G 7.5 μg , pMDLg/pRRE 15 μg and pRSV-Rev 10 μg) and 165 μL lipofectamine 2000. Before transfection, 10 mL of culture medium was removed from the culture dishes, and complexes of plasmid DNA and Lipofectamine 2000 were added evenly onto the medium. Six hours after transfection, the plates were washed twice with PBS and 20 mL of fresh medium was

added. The supernatant was collected 48 hours posttransfection and cell debris was removed by centrifugation at $6000\times g$ for 10 minutes. Supernatant containing viruses was tittered using a p24 kit (ZeptoMetrix, Buffalo, NY) and was either used directly or stored at -800°C .

Activation of CD8+ T Cells by Anti-CD3/CD28 Beads

At day 0, negative-selected or positive-selected CD8+ T cells were washed twice with PBS and activated by anti-CD3/CD28 beads (Invitrogen, Carlsbad, CA) (bead to CD8+, 3:1) overnight. At day 1 and day 2, 1-mL lentiviral vector were applied in the presence of $10\text{-}\mu\text{g/mL}$ protamine sulfate, and the plates were centrifuged at $1000\times g$, 32°C for 2 hours. Six hours after the second transduction, the medium was replaced with AIM-V containing 5% human serum, 300 IU/mL IL-2. At day 5, cells were transferred to 6-well plates and split. At day 12, 1×10^6 cultured cells were either stimulated with anti-CD3/CD28 beads in a ratio of bead to cells (3:1) or with PBMC feeder cells at a ratio of feeder to cells (100:1) in the presence of 30 ng/mL OKT3 and 300 IU/mL IL-2 for 12 days and maintained with fresh culture medium and vessels as needed.

Plate-bound OKT3 Activation of Positive-selected CD8+ T Cells

Four milliliters of PBS containing $1\text{ }\mu\text{g/mL}$ OKT3 was added to nontissue culture 6-well plates overnight at 4°C , and blocked with PBS containing 2% FBS for 30 minutes at room temperature. After removing the supernatant, the plates were immediately used for stimulation. At day 0, positively selected CD8+ T cells were washed twice with PBS, and 5×10^6 cells in AIM-V medium containing 5% FBS (300 IU/mL IL-2) were added to wells of plate-bound OKT3 plates followed by $1000\times g$ spinoculation for 10 minutes. At day 1, cells were washed twice with PBS and transduced by spinoculation of 1×10^6 cells per well in 24-well plates. After spinoculation, plates were put back into a CO_2 incubator. Six hours after transduction, feeder cells from 3 donors PBMC were irradiated (40 Gy) and added to wells of 24-well plates containing transduced cells in a ratio of feeder to CD8+ (10:1) in the presence of OKT3 (30 ng/mL) and IL-2 (300 IU/mL). Plates were put back into a CO_2 incubator. At day 2, the medium was removed and replenished with AIM-V containing 5% human serum, 300 IU/mL IL-2 for extended culture. At day 4 or day 5, cells were transferred to 6-well plates and passaged as needed to maintain a cell density of 0.5 to $2.0\times 10^6/\text{mL}$. For transduction of clinical-grade CD8+ T cells, the transduced cells were transferred to 6-well plates, followed by 75 cm^2 and then 175 cm^2 flasks without discarding any cells during the 12 days in vitro culture.

Fluorescence-activated Cell Sorter (FACS) Analysis

Cell surface expression of gp100 (154) TCR, MART-1 TCR, CD3, CD4, CD8, CD27, CD28, CD62L, CD45RO, CD45RA, CD70, CD56, CCR7, and NKG2D was measured using fluorescein isothiocyanate (FITC), APC, or phycoerythrin (PE)-conjugated antibody or custom designed tetramers.⁸ All FACS data were analyzed using FlowJo 8.1.1 software (FlowJo, Ashland, OR).

Measurement of Lymphocyte Reactivity to Antigen

Transduced CD8⁺ T cells (1×10^5) were cocultured with melanoma lines (1×10^5) in a final volume of 0.2 mL in each well of a round-bottom 96-well plate. Cell culture supernatants were harvested and assayed 16 hours later for IL-2 and IFN γ by ELISA (Pierce Endogen, Rockford, IL). The ability of transduced CD8⁺ T cells to lyse HLA-A2⁺ matched melanoma cells was evaluated using a ^{51}Cr assay as described.²³

RESULTS

Limitations of Anti-CD3/CD28 Beads for Activation of CD8⁺ T Cells

On the basis of published protocols for lentiviral vector-mediated gene transfer and expansion, we first tested the potential of anti-CD3/CD28 beads for activation of CD8⁺ T cells.^{10,24} The lentiviral vectors used contained an anti-MART-1 TCR, as reported earlier.⁸ We carried out 2 rounds of transduction using CD8⁺ T cells purified by “negative-selection,” and activated using anti-CD3/CD28 beads (Fig. 1A). Transgene expression and purity of CD8⁺ T cells were evaluated at day 7, day 12, and at day 24 after a second stimulation (S2) at day 12. The purity of negatively selected CD8⁺ T cells immediately after selection was more than 95% (data not shown). The expression of MART-1 TCR detected by tetramer was up to 89% in 3 donors tested (Fig. 1B). Starting at day 7, we observed a gradual accumulation of CD4⁺ T cells in all 3 donors tested that was most pronounced for donor 1 when measured at day 12 (Fig. 1B). The transduced cells expanded an average of 50-fold by day 12 and showed specific IFN- γ secretion after antigen exposure (Fig. 1C on left). To compensate for the relative low cell numbers at day 12, we initiated a second stimulation using anti-CD3/CD28 beads, or PBMC feeder cells.¹⁷ We observed in 3 donors that anti-CD3/CD28 beads expanded cells 28-fold whereas the use of feeder cells robustly expanded cells more than 2000-fold (Fig. 1C on right). It is interesting to note that after S2 anti-CD3/CD28 bead-activated cells maintained a higher level of TCR expression in donor 2 and donor 3 than the feeder group, but they exhibited a lower potential for IFN- γ induction (Fig. 1C on right). In addition, donor 1 reverted to a CD4⁺ dominant phenotype after a second round of bead stimulation. All T cells after S2 lost CD62L expression and exclusively displayed an effector memory phenotype (See Supplemental Digital Content 1, <http://links.lww.com/JIT/A55>).

Feeder Cells Were Superior to Anti-CD3/CD28 Beads in Supporting the Growth of TCR Gene-engineered CD8⁺ T Cells

The results in Figure 1 showed that using anti-CD3/CD28 beads to activate negatively selected CD8⁺ T cells resulted in preferential expansion of CD4⁺ T cells and failed to efficiently propagate CD8⁺ T cells in a second stimulation. Furthermore, for clinical application, negative selection of CD8⁺ T cells is mediated by a cocktail of antibodies that are not available for clinical use. In current clinical applications, CD8⁺ T cells are obtainable through positive selection using Good Manufacturing Process (GMP) approved CD8⁺ microbeads (Miltenyi Biotec). To develop lentiviral vector-mediated transduction of CD8⁺ T cells for clinical applications, we compared positive selection to isolate CD8⁺ T cells with negative selection and determined the phenotype of cells day 1 after selection. In general, both methods yielded a pure CD8⁺ population (>93%), and exhibited a similar

phenotype. However, although positive selection yielded slight higher percentage of CD8⁺ cells there were 4% to 9% of CD3-CD8⁺ cells observed in the CD8⁺ T-cell population obtained by positive selection (second column in Figs. 2A, B indicated by arrows). This CD3-CD8⁺ population has been described as belonging to NK cells.²⁵

To evaluate the potential utility of anti-CD3/CD28 beads to activate and expand positively selected CD8⁺ T cells, we compared the efficacy of using anti-CD3/CD28 beads and feeder cells from PBMC in supporting gene delivery and growth. In Figure 3A, the CD8⁺ T cells from both positive and negative isolation methods were activated with beads as described earlier or with feeder cells in the presence of anti-CD3 antibody. The next day, cells from both activation methods were transduced with a lentiviral viral vector harboring the anti-MART-1 TCR. After 12 days as shown in Figure 3B, anti-CD3/CD28 beads provided suitable activation for lentiviral vector-mediated transduction (MART-1 positive cells up to 86%) but failed to efficiently expand CD8⁺ T cells using either selection method. In contrast, activation using feeder cells yielded a slightly less degree of transduction (MART-1 positive cells up to 53%) but mediated a robust expansion of CD8⁺ T cells using either selection method (Fig. 3B, lower panel). Consistent with results shown in Figure 1, although the anti-CD3/CD28 beads yielded a higher percentage of MART-1 positive cells than the CD8⁺ T cells from feeder cells, we observed similar levels of IFN- γ release in coculture assays (Fig. 3C on left) and CD8⁺ T cells expanded by feeder cells produced more IL2 after coculture (Fig. 3C on right).

Plate-bound Anti-CD3 Activation Provided an Ideal Platform for Lentiviral Vector-mediated Transduction and Expansion of CD8⁺ T Cells

The goal of this study was to develop a suitable activation platform for CD8⁺ T cells that facilitated lentiviral vector-mediated transgene expression and robust expansion of cells for clinical applications of adoptive immunotherapy. As an alternative to stimulation methods based on CD3/CD28 beads or feeder cells, we tested immobilized anti-CD3 antibody (OKT3) as an activation method followed by transduction and then expansion using PBMC feeders (Fig. 4A). CD8⁺ T cells were stimulated with plate-bound OKT3 then subject to a single transduction the next day. Six hours posttransduction, irradiated feeder cells (feeders cells to CD8⁺ T cells, 10:1) were applied to expand the transduced cells in the presence of OKT3 and IL-2. The cells were maintained for 12 days and analyzed (Fig. 4B).

The cells with or without OKT3 overnight stimulation showed similar viability and expression of differentiation markers except for internalization of CD3 antigen (Supplemental Digital Content 2A, 2B, column 2, Supplemental Digital Content 2, <http://links.lww.com/JIT/A56>). We observed robust transgene expression (up to 80%) and a high purity of CD8⁺ T cells (up to 94%) after 12 days of growth supported by feeder cells. These cultures expanded up to 590-fold and more than 50% of cells exhibited a CD45RO⁺CD62L⁺ central memory-like phenotype (Fig. 4B, column 4). We tested an additional 4 donor CD8⁺ T cells and in 3 independent experiments, the cells underwent 246 $\pm$ 174-fold expansion, the average MART-1 TCR expression was 77% $\pm$ 8.9%, and a majority of cells (61% $\pm$ 7.6%) were CD62L positive (data not shown). The positively selected CD8⁺ T cells activated by plate-

bound OKT3 followed by transduction and expansion using feeder cells were functionally active as evaluated by coculture with antigen matched melanoma lines (Fig. 4C).

To further confirm that the superiority of plate-bound OKT3 plus feeder stimulation to anti-CD3/CD28 beads stimulation and expansion of CD8⁺ T cells, we set-up a comparison experiment as shown in Figure 5A. In this head-to-head comparison, the anti-CD3/CD28 beads again failed to expand CD8⁺ T cells efficiently (21-fold compared with 192-fold for plate-bound OKT3, Fig. 5B). We once more observed that although the CD3/CD28 stimulated CD8⁺ T cells yielded enhanced gene transfer (as judged by tetramer staining), this did not translate into greater IFN- γ induction after coculture (Fig. 5C).

Genetic Modification of CD8⁺ T Cells for Clinical Adoptive Immunotherapy

For potential clinical application, we tested the clinical-scale isolation of CD8⁺ T cells using the CliniMACS separation system (Miltenyi Biotec). A routine CliniMACS separation of PBMC derived from leukapheresis yielded about 60 million positively selected CD8⁺ T cells using GMP approved clinical protocols. The selected CD8⁺ T cells contained about 8% of CD3-CD8⁺ NK cells, and actively responded to plate-bound OKT3 stimulation as shown by decreased CD3⁺ staining (Supplemental Digital Content 2C, 2nd column, Supplemental Digital Content 2, <http://links.lww.com/JIT/A56>). On the basis of our laboratory-scale procedures, the cells were stimulated overnight on OKT3-coated plates and were transduced the next morning. Six hours after transduction, feeder cells were added and the T-cell cultures were maintained by adjusting growth medium and culture vessels as needed for 12 days total ex vivo growth. The data (Fig. 6A) showed that we could efficiently transduce CD8⁺ T cells isolated under clinical conditions with TCRs reactive with MART-1, or gp100 and with GFP up to 90%. The transduced CD8⁺ T cells showed an exponential growth occurring between days 6 to day 11, resulting in up to a 600-fold expansion over 12 days (Fig. 6A). The final cell product was composed primarily of CD8⁺ T cells (about 95%) with a few NK-like cells (Fig. 6B, column 2), and showed central memory-like phenotype defined by CD45RO⁺ and CD62L⁺ (Fig. 6B, column 4) and high levels of the CD27/CD28 coreceptors (Fig. 6B, last column). Using coculture assays, we observed specific cytokine induction and lysis only in cells transduced with TCRs (MART-1 or gp100) not in cells expressing GFP (Figs. 6C, D).

DISCUSSION

Antigen-specific CD8⁺ T cells can be used to treat cancer and prevent infections in humans.^{2,26–28} The genetic modification of CD8⁺ T cells using antitumor T-cell receptors is a promising approach for the adoptive cell therapy (ACT) of patients with cancer. Our rationale for using CD8⁺ T cells as targets for gene transfer are that most class-I restricted TCRs are CD8-dependant and do not function well when transferred to CD4⁺ T cells,²¹ and it has been reported that the transfer of CD4⁺ Tregs has the potential to decrease the effectiveness of ACT in animal models.²⁹ In human gene therapy applications, lentiviral vectors (LVV) possess advantages compared with γ -retroviral vectors in several aspects including the ability to transduce nondividing cells, resistance to gene silencing, and a potentially safer integration profile.^{30,31} The published clinical protocols using lentiviral vectors for genetic

modification of T cells relies on anti-CD3/CD28 bead stimulation.²⁴ Although anti-CD3/CD28 beads provided a suitable activation for lentiviral vector-mediated gene delivery and expansion of CD4+ T cells for anti-HIV-1 gene therapies,²⁴ CD8+ T cells are not efficiently expanded.¹⁵ Alternatively, irradiated feeder cells from PBMC in the presence of anti-CD3 antibody have been shown to be an effective approach to expand tumor or virus specific T-cell clones,¹⁶ TIL from melanoma tissues,¹⁷ and PBMC from patients with advanced metastatic melanoma.^{7,18}

Although anti-CD3/CD28 beads provide a suitable activation stimulus for lentiviral vector-mediated transduction of positive-selected CD8+ T cells, the insufficient expansion of CD8+ T cells limits its clinical application. To maximize the production of genetically engineered T cells, we found the optimal protocol to be; plate-bound OKT3 for activation of positive-selected CD8+ T cells followed by transduction and then the addition of feeder cells for expansion. The feeder cells used in all these experiments were pools of 3 randomly chosen patient PBMC preparations. The 12-day fold expansion results using feeder-based propagation of transduced cells ranged from 114 to 645-fold (mean 326±206) possibly owing to donor-to-donor or feeder cell pool variations.

Positively selected CD8+ T cells activated by plate-bound OKT3 exhibited a similar phenotype compared with cells without OKT3 stimulation except for internalization of the CD3 antigen that can be used as a marker to monitor T-cell activation prior to lentiviral vector transduction. After 12 days in vitro culture, these highly transduced CD8+ T cells could be expanded up to 600-fold and exhibited a less-differentiated phenotype illustrated by high percentages of CD62L, CD28 and CD27 cells. One of the characteristics of CD8+ T cells generated in this study was that they exhibited high levels of CD62L, which is consistent with a central memory-like phenotype. CD62L acts as a ‘‘homing receptor’’ for leukocytes to enter lymphoid tissues through high endothelial venules. CD62L is commonly found on the cell surfaces of naïve T cells and is needed to facilitate the entry of naïve cells to lymph nodes where they encounter antigen. Central memory T cells, which have earlier encountered antigen, also express CD62L, which similarly permits these cells to localize to secondary lymphoid organs leading to recall antigen-mediated proliferation and eventual lymph node release and trafficking to sites of disease. Animal studies showed that the initial interaction of T cells with secondary lymphoid tissues rather than tumor sites, facilitated the eradication of large established tumors.⁶ Moreover, compared with effector T cells, central memory T cells have an intrinsic capacity enabling them to survive long-term after adoptive transfer and to populate the memory cell pool.³ All of these reports suggest that the recruitment of central memory T cells may be beneficial for adoptive immunotherapy to treat cancer and prevent infections.

Last, to further augment the potential application of this methodology we chose CD8+ T cells isolated using reagents certified for clinical use. By a routine separation process, we could obtain 60 million CD8+ T cells from PBMC using the CliniMACS system, and these cells could be readily activated and transduced with 3 different lentiviral vectors with transduction efficiencies up to 90%. Moreover, large numbers ($>1 \times 10^9$) transduced CD8+ T cells can be rapidly (<12 d) obtained and these T cells exhibited a less-differentiated central memory-like phenotype and showed antitumor biologic activities. Although there are

issues with the continuing manufacture of clinical-grade OKT3, our data support the use of other sources of anti-CD3 antibody, which have similar activities. The methodology described here represents a simple and efficient approach to genetically engineer CD8+ T cells with antitumor specificity for adoptive immunotherapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

We thank the FACS laboratory and the TIL laboratory in the Surgery Branch, National Cancer Institute, for providing technical support and maintenance of tumor cells from patients. This work is supported by the Intramural Research Program of the National Institutes of Health, National Cancer Institute, Center for Cancer Research.

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FIGURE 1.

Lentiviral vector-mediated transduction of CD8⁺ T cells activated by anti-CD3/CD28 beads. A, Schematic illustration of experimental design. Negative-selected CD8⁺ T cells were activated using anti-CD3/CD28 beads at a bead to cell ratio of 3:1 using 1×10^6 cell per well of a 24-well plate. One day later, cells were washed twice with PBS and transduced with lentiviral vector supernatant as described in methods. Transduced cells were fluorescence-activated cell sorter (FACS) analyzed at day 7 and day 12 posttransduction. At day 12, cells were subject to a second expansion (S2) using either anti-CD3/CD28 beads or PBMC feeder cells supplemented with 30 ng/mL OKT3. At day 24 poststimulation (S2d12), cells were analyzed by FACS and their function tested. B, T-cell antigen receptor (TCR) expression after lentiviral vector-mediated transduction. CD8⁺ T cells were from 3 donors PBMC (d1, d2, and d3), were analyzed for transgene TCR expression by staining with MART-1 tetramer and purity of CD8⁺ T cells was determined by anti-CD8 antibody. C, Functional analysis of transduced CD8⁺ T cells. The cells were counted at day 12 (left) and day 24 (right) posttransduction and the actual fold expansion determined. IFN- γ induction was carried out by coculture with melanoma tumor lines Mel526, Mel 624 (HLA-A2⁺, MART-1⁺) and Mel

888, Mel 938 (HLA-A2–, MART-1+). Plotted were the means of triplicate values determined after overnight coculture.

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FIGURE 2.

Phenotypic analysis of CD8⁺ T cells from negative or positive selection. A and B, Phenotype of negatively or positively selected CD8⁺ T cells at day 1. CD8⁺ T cells from 2 donors (d7 and d8) were obtained by negative or positive selection and cultured in AIM-V medium containing 300 IU/mL IL-2 overnight. The phenotype of cells were evaluated by FACS using a panel of antibodies indicated on top of each image. The arrows in second column on “positive selection” groups indicate populations of CD3-CD8⁺ NK cells.

FIGURE 3.

Feeder cells from PBMC support both gene delivery and robust expansion of CD8⁺ T cells derived from negative or positive selection. A, Schematic illustration of anti-CD3/CD28 beads or feeder cells-mediated activation and transduction. CD8⁺ T cells from 2 donors (d7 and d8) were obtained by negative or positive selection and activated by anti-CD3/CD28 beads or feeder cells (in the presence of 30 ng/mL OKT3) overnight. The next day the cells were washed twice and transduced with lentiviral vector by spinoculation in the presence of protamine sulfate, and cultured for 12 days. B, At day 12, the transgene expression and phenotype of cells were evaluated using a panel of antibodies as indicated. The fold expansion was listed on right of each group. Top panel represents the anti-CD3/CD28 beads activated CD8⁺ T cells, and lower panel represents feeder cells activated CD8⁺ T cells. C, At day 12, the function of transduced cells activated by anti-CD3/CD28 beads or feeder cells was tested for IFN γ (left) and IL2 (right) by coculture with melanomalines, where d7- and

d8-indicate donor-specific CD8⁺ T cells from negative selection, whereas d7⁺, d8⁺ indicate CD8⁺ T cells from positive selection.

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FIGURE 4.

Lentiviral vector-mediated transduction of CD8⁺ T cells using plate-bound OKT3 activation.

A, Schematic illustration of plate-bound OKT3 activation and transduction. B, Phenotypic analysis of CD8⁺ T cells activated by plate-bound OKT3. Positive-selected CD8⁺ T cells were activated by plate-bound OKT3 overnight, and the next day cells were washed twice with PBS and transduced by spinoculation as described in methods. Six hours after transduction, feeder cells (feeders to CD8, 10:1) were added to 24-well plates and briefly resuspended by gentle mixing. At day 2, cell media was replenished with fresh media, and at day 4, cells were transferred to 6-well plates. At day 12, the transgene expression and phenotype of cells were evaluated using a panel of antibodies as indicated. The number representing fold expansion was listed on right. C, Cytokine production by transduced CD8⁺ T cells. At day 12, the function of transduced cells activated by aAPC or feeder cells was tested for cytokine induction by coculture with melanoma lines. d4, d5 indicate CD8⁺ T cells from donor 4 and donor 5.

FIGURE 5.

Head-to-head comparison of plate-bound OKT3 to anti-CD3/CD28 beads in the activation and expansion of CD8⁺ T cells. **A**, Schematic illustration of plate-bound OKT3 activation or anti-CD3/CD28 beads. **B**, Phenotypic analysis of CD8⁺ T cells activated by plate-bound OKT3 and anti-CD3/CD28 beads. Positive-selected CD8⁺ T cells obtained from donor 6 (d6) were activated by plate-bound OKT3 or anti-CD3/CD28 beads overnight, and then transduced with the anti-MART-1 TCR vector. Six hours after transduction, feeder cells were added to the plate-bound OKT3 activated CD8⁺ T cells, for expansion. At day 12, the transgene expression and phenotype of cells were evaluated using a panel of antibodies. The number representing fold expansion was listed on right. **C**, Cytokine production by transduced CD8⁺ T cells. At day 12, the function of transduced cells was tested for IFN- γ cytokine induction following coculture with melanoma lines.

FIGURE 6.

Efficient transduction and robust expansion of clinical-grade CD8⁺ T cells. **A**, Lentiviral vector-mediated transgene expression and expansion. CD8⁺ T cells from donor 6 (d6) were purified using the CliniMACS separation system from Miltenyi Biotec. CD8⁺ T cells were activated using plate-bound OKT3 for 1 day, and transduced the next day by spinoculation as described in methods using vectors containing MART-1 or gp100 TCRs or green fluorescent protein (GFP) as control. Six hours after transduction, PBMC feeder cells were added (feeder cells to CD8⁺, 10:1) and mixed. The cells were cultured in vitro for 12 days and the expression of 2 TCRs and GFP was measured by FACS (left side). The growth curve of DMF5 TCR transduced cells was plotted in the middle panel and the fold expansion of these transduced cells was calculated on right. **B**, Phenotypic analysis of transduced CD8⁺ T cells at day 12. A panel of differentiation markers were used as denoted on top of each FACS image. **C**, Cytokine induction. Transduced CD8⁺ T cells were cocultured with

melanoma lines, and cytokine production of IFN- γ and IL-2 was measured by ELISA. D, Specific lysis of melanoma lines. The specific lytic activities of transduced CD8+ T cells were determined by coculture with ^{51}Cr -labeled tumor cells at the indicated E: T ratios. The percent cell lysis was calculated using the formula $[(\text{specific release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})] \times 100$. Results representing mean of triplicate cultures were plotted.

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