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***In vitro* generated anti-tumor T lymphocytes exhibit distinct subsets mimicking *in vivo* antigen experienced cells**

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Abstract

The T lymphocyte pool can be sub-divided into naïve (Tn), effector memory (Tem), and central memory (Tcm) T cells. In this study, we characterized *in vitro* short-term cultured anti-tumor human T lymphocytes generated by lentiviral transduction with an anti-tumor antigen TCR vector. Within two weeks of *in vitro* culture, the cultured T cells showed a Tcm-like phenotype illustrated by a high percentage of CD62L and CD45RO cells. When the cells were sorted into populations that were CD45RO+/CD62L− (Tem), CD45RO+/CD62L+ (Tcm) or CD45RO^{low}/CD62L+ (Tn) and co-cultured with antigen-matched tumor lines, the magnitude of cytokine release from these populations for IFN γ (Tn<Tcm<Tem) and IL-2 (Tn>Tcm>Tem) mimicked the types of immune cell responses observed *in vivo*. In comparing cell-mediated effector function, Tn were found to be deficient (relative to Tcm and Tem) in the ability to form conjugates with tumor cells and subsequent lytic activity. Moreover, analysis of the gene expression profiles of the *in vitro* cultured and sorted T cell populations also demonstrated patterns consistent with their *in vivo* counterparts. When Tcm and Tem were tested for the ability to survive *in vivo*, Tcm displayed significantly increased engraftment and persistence in NOD/SCID/ γ c^{−/−} mice. In general, a large percentage of *in vitro* generated anti-tumor T lymphocytes mimic a Tcm-like phenotype (based on phenotype, effector function, and increased persistence *in vivo*), which suggests that these Tcm-like cultured T cells may be optimal for adoptive immunotherapy.

Keywords

Gene therapy; Lentiviral vector; T cell receptor; Central memory cells; Effector memory cells; Tumor immunity

Introduction

Adoptive cell therapy (ACT) using tumor-infiltrating lymphocytes (TIL) is a potent treatment for patients with metastatic melanoma, and can mediate objective responses in 50–70% of patients (1–3). However, not all patients have pre-existing tumor reactive T lymphocytes, and these tumor reactive lymphocytes are generally only found in melanoma patients, which limits the broad application of this approach. The transduction of peripheral blood lymphocytes (PBL) with genes encoding anti-tumor TCRs using γ -retroviruses readily renders autologous PBL from any patient into tumor killing T lymphocytes *in vitro*(4). Administration of anti-MART-1 TCR engineered PBL in patients with advanced metastatic

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melanoma resulted in a 30% objective response rate (5,6), and holds the promise for treatment of other types of cancer. Genetic modification of T cells *in vitro* with anti-tumor TCRs, followed by robust *in vitro* expansion, may avoid immune tolerance observed *in vivo* and can generate sufficient numbers of specific anti-tumor T cells for patient treatment.

For TCR gene therapy, current protocols using γ -retroviral vectors require T cells to be actively dividing for efficient gene integration into cellular DNA. The need for fully dividing T cells limits the number of cells that can be transduced, and often requires a second rapid expansion (REP) in order to generate enough cells for clinical application. However, this second expansion causes cells to become fully differentiated and exhibit an effector memory phenotype that may impede *in vivo* persistence (7) and *in vivo* tumor killing efficacy (8). Compared to γ -retroviral vectors, lentiviral vectors efficiently transduce non-dividing cells. The minimal requirement for lentiviral vector-mediated transduction of quiescent T cells is that T cells enter into the G1 phase of the cell cycle. Following anti-CD3 activation, quiescent T cells easily move into the G1 phase within hours (9,10). The use of anti-CD3/CD28 beads provided a simple activation method for lentiviral vector mediated transduction and transgene expression (9,11), and a clinical-scale transduction protocol has been reported to yield $> 1 \times 10^{10}$ transduced T cells in a minimal culture period (9).

In humans, the T lymphocyte pool can be sub-divided into naïve antigen inexperienced cells (T_n), and following antigen encounter into effector memory (T_{em}), and central memory (T_{cm}) T cells (12–15). T_{cm} cells are thought to contain a long-lived memory cell population displaying a capacity for self-renewal associated with high levels of phosphorylated transcription factors (16). Recent studies suggest that there are additional subsets of CD8⁺ T memory cells in mice (17) and in humans (18). Central memory CD8⁺ T cells and effector memory CD8⁺ T cells have been identified in humans and animals, and can be distinguished in part by the expression level of CCR7, CD62L (19) and the secretion of cytokines (15). Animal studies suggest that central memory T cells not only survive longer after ACT (7), but also confer superior anti-tumor reactivity compared to effector memory T cells, and can lead to the eradication of large established tumors (20).

The generation of an active population of memory T cells is pivotal for effective vaccine and cell-based therapies to fight infectious diseases and cancer (21). The phenotypic and functional characterization of immune T cells after antigen challenge *in vivo* has been well studied (15,22). However, the immune-related characteristics of *in vitro* generated anti-tumor antigen TCR engineered T cells remains to be elucidated. In this report, we systematically analyzed *in vitro* TCR gene-engineered T lymphocytes for their phenotype, function, and *in vivo* engraftment to determine the optimal type of cell for use in adoptive cell therapy.

Materials and methods

Cell culture

PBL used in this study were obtained from healthy donors or metastatic melanoma patients seeking treatments at the Surgery Branch, National Cancer Institute under approved clinical protocols. Briefly, PBL were collected by leukapheresis, and lymphocytes were separated by Ficoll/Hypaque cushion centrifugation, washed in HBSS and resuspended at a concentration of 1×10^6 /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). Melanoma cell lines included MART-1 positive HLA-A2⁺ 526 and MART-1 positive HLA-A2⁻ 938. 293T cells (ATCC, Manassas, VA) were cultured in DMEM supplemented with 10% FCS, 100 U/ml penicillin/streptomycin, 2 mM L-glutamine (Invitrogen). All cell lines were cultured at 37°C in a 5% CO₂ humidified incubator.

Vector construction and lentivirus preparation

The lentiviral constructs utilized 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(23). Woodchuck hepatitis virus response element (wPRE) was replaced with the truncated form oPRE, where the residual X protein and its promoter were deleted (24). The oPRE sequence was synthesized and 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 to yield vector pLLV.GFP.oPRE. A lentiviral vector expressing the gp100 TCR alpha and beta chains of DMF5 TCR targeting melanoma antigen MART-1(25) were previously described (9,26,27). All the constructs are confirmed by restrictive enzyme digestion and sequencing. For 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 h 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 (Invitrogen). 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 h post-transfection and cell debris was removed by centrifugation at $6000 \times g$ for 10 min. Supernatant containing viruses was tittered using a p24 kit (ZeptoMetrix, Buffalo, NY) and was either used directly or stored at -80°C .

The generation of anti-tumor T lymphocytes and FACS analysis/sorting

For cell preparation, briefly PBL were activated using anti-CD3/CD28 beads overnight, and next day the cells were transduced with lentiviral vector harboring anti-tumor TCR by spinoculation as previously described in detail (9). Six hours post-transduction the cells were transferred to 75 cm² flask and maintained below concentration of $10^6/\text{ml}$. Cell surface expression of CD3, CD4, CD8, CD27, CD127, CCR7, CD28, CD70, CD95, CD137, CD57, CD62L and CD45RO was measured using fluorescein isothiocyanate (FITC), Allophycocyanin (APC), phycoerythrin (PE) PE-CY-7, or APC-CY-7, conjugated antibodies (BD Biosciences, San Jose, CA). MART-1:27–35 tetramer (PE or APC) was used (iTag MHC Tetramer, Beckman Coulter, Fullerton, CA) to verify TCR gene transfer. Immunofluorescence staining was analyzed as the relative log fluorescence of live cells, determined using a FACScan flow cytometer (BD). A combination of forward angle light scatter and propidium iodide staining was used to gate out the dead cells, and 1×10^5 cells were analyzed. All FACS data was analyzed using FlowJo 8.1.1 software (Tree Star Inc., Ashland, OR). For cell sorting, 14 day-cultured cells were washed twice with PBS and stained with anti-human CD8– or CD3-PE-Cy-7, CD45RO-FITC, CD62L-APC (BD Biosciences,) and propidium iodide (PI) (50 µg/ml). Before sorting, the stained cells were passed through 40 µm Nylon cell strainer (BD). The cells were either gated on CD8 or CD3 subsets depending on the specific experiment, and using differentiation markers CD45RO and CD62L to yield CD62L–/CD45RO+ (Tem), CD62L+/CD45RO+ (Tcm) and CD62L+/CD45RO– (Tn) populations. The sorting was performed on a FACS Aria I cytometer (BD) equipped with 2 laser system (488 nm blue laser and 633 nm red laser), the cells were passed through 70 µm nozzle under pressure of 70 psi. The cytometer was driven by BD FACSDiva version 6.1.3 software.

Illumina gene expression array

Total RNA from sorted Tem, Tcm and Tn CD8 sub-populations was extracted, and 200ng of total RNA was used to generate biotin-labeled cDNA using Illumina TotalPrep RNA amplification kit (Ambion, Austin, TX). Biotin labeled cDNA (750 ng) was hybridized to

Sentrix BeadChip Array for Gene Expression (Human Ref-12 V2, Illumina, San Diego, CA) and incubated at 58°C for 16–20 h in an Illumina hybridization oven with rocker speed at 5. Beadchips were washed and stained according to Illumina's protocol. Arrays were scanned by Illumina chip scanner, and images analyzed by Bead Studio (Illumina, San Diego, CA). Data were exported and processed using Genespring GX 11 (Agilent, Santa Clara, CA). Differentially regulated genes between different sub-populations were identified by statistical significance $p < 0.05$ and fold change > 2 . Significantly regulated genes were subjected to further analysis using Ingenuity Pathway software (Ingenuity, Redwood City, CA).

Effector-Target conjugate formation assay

The assay for effector-target cell conjugate formation has been previously reported (28). Briefly, melanoma line 526 was labeled with CFSE at a concentration of 1 μ M using CFSE cell proliferation kit (Invitrogen) according to product manual. 1×10^7 melanoma 526 cells were labeled, and the cells were cultured for 48 h prior to use. Lentiviral TCR-vector-transduced PBL (14 days in culture) were sorted as described above. The co-culture was initiated by combining 3×10^5 each of target and effector cell in 14 ml polypropylene round-bottom tubes (BD) by centrifugation at 137g for 10s. At the end of incubation, the cells were stained with CD3 PerCP, CD8 APC and MART-1 PE tetramer to analyze for T cell:tumor cell conjugates by FACS. FACS data was analyzed using FlowJo software. The percentage of effector-target conjugate formation was determined using double positive cells divided by CFSE labeled tumor cells, the statistic analysis was based on mean $\pm$ SD from triplicate samples.

Measurement of lymphocyte reactivity to antigen

Transduced PBL effector cells (1×10^5) were co-cultured 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 h later for IFN γ and IL2 by ELISA kit (Pierce Endogen, Rockford, IL). The culture supernatants were diluted to be in the linear range of the assay. Results represent mean $\pm$ SD of triplicate cultures. The ability of transduced PBL to lyse melanoma tumor lines was evaluated using a ^{51}Cr assay as described(29). Briefly, 10^6 tumor cells were labeled for 1 h at 37 °C with 100 μ Ci of ^{51}Cr (GE Healthcare, Piscataway, NJ). Labeled target cells (2×10^3) were co-cultured with effector cells at the ratios indicated for 4 h at 37 °C in 0.15 ml of medium. Harvested supernatants were counted using a MicorBeta TriLux instrument (Perkin Elmer, Waltham, MA). Total and spontaneous ^{51}Cr release was determined by incubating 2×10^3 labeled target cells in either 2% SDS or medium alone for the above conditions respectively. Each data point was determined as a mean of quadruplicate wells. The percentage of specific lysis was calculated as indicated in figure legend.

Engraftment of Tem and Tcm in NOD/SCID/ γ c $^{-/-}$ mouse model

Sorted CD3 Tem and Tcm sub-populations were counted and suspended in PBS, 1.7×10^6 cells were injected intravenously into NOD/SCID/ γ c $^{-/-}$ mice without supplement of IL2. Thirty-seven days later, mice were sacrificed and T lymphocytes were isolated from spleen, lymph nodes (LN) and lung. T cells from lymph node (LN) spleen were lysed with ACK lysing buffer (BioWhittaker) followed by two PBS washes. T cells from lung were minced and tissue debris was removed. Lymphocyte separation medium (MP Biomedicals) was used to purify T cells by centrifugation at 2000g for 20 min. After collecting lymphocytes, and washing twice, the cells were quantitated and subject to FACS analysis.

Results

Phenotype and activity of *in vitro* generated anti-tumor T lymphocytes

Using overnight activation with anti-CD3/CD28 beads followed by lentiviral vector-mediated transduction (Figure 1A), engineered cells from three donors were expanded and harvested at day 14, and the phenotype of CD8⁺ T cells was analyzed using differentiation markers CD45RO and CD62L. As shown in Figure 1B, the Tcm cells were the least abundant at day 0, while this phenotype was the most plentiful at day 14 following transduction and *in vitro* culture. The phenotype of the three distinct sub-populations was further analyzed (Figure 1C). The differentiation markers CD27, CCR7 and CD127/IL7R were preferentially expressed on the Tn population, while the differentiation marker CD70 showed the opposite pattern, i.e., the lowest expression occurred on the Tn population. We did not observe significant differences in other markers analyzed (CD95, CD137, CD28 and CD57).

To verify the functional activity of these gene-engineered cells, PBL from two donors were transduced and expanded for 14 days, and the TCR expression and the phenotype of T cells were analyzed using MART-1 Tetramer and differentiation markers (Figure 2A). The fold expansion within 14 days was illustrated on right. The specific TCR used for transduction is restricted for HLA-A0201 and renders these cells reactive against HLA-A2 expressing tumors (i.e., melanoma line 526). The transduced cells demonstrated effector functions including cytokine secretion (IFN γ and IL-2, Figure 2B) and specific tumor cell lysis (Figure 2C). To determine the reproducibility of this genetic modification protocol, we tested a total of six donors in three independent experiments (Supple Table 1). In summary, the PBLs from melanoma patients could be efficiently transduced with an anti-tumor TCR (70.6 ± 18.4), robustly expanded (568.7 ± 355.8), and the majority of these T cells showed Tcm-like phenotype.

Function of *in vitro* cultured TCR gene-engineered T lymphocytes

To determine the functional activity of the phenotypically distinct T cell sub-populations, CD8⁺ Tem, Tcm and Tn (defined by CD45RO and CD62L) cells were sorted for analysis of effector function (Figure 3A). The purity of sorted cells and the expression of anti-tumor TCR MART-1 on each subset was as shown (Figure 3B). These sorted cells when co-cultured with melanoma lines (526, HLA-A2⁺; 938, HLA-A2⁻) and shown to exhibit distinct biological activities (Figure 3C). Tem demonstrated the greatest potential for IFN γ synthesis followed by Tcm and Tn, while the production of IL-2 displayed the opposite trend. The lytic activities correlated with IFN γ induction, where the Tn sub-population had minimal specific lysis (Figure 3C).

To further determine the intrinsic differences of these sub-populations, we tested the potential of these sub-populations to form conjugates with the HLA matched tumor line 526 (the positive target cell for TCR recognition). In this assay, CFSE labeled target cells were co-cultured with CD8 positive Tem, Tcm and Tn cells. 30-min later, the co-cultured cells were stained with anti-CD8 antibody and subject to FACS analysis and quantitation (Figure 4). After normalization, the Tn sub-population displayed the lowest potential to form effector-target conjugates, while Tem and Tcm showed similar activities in forming effector-target conjugate (Figure 4). The ability to form effector-target conjugates was consistent with the lytic activity shown in Figure 3. Interestingly, while the TCR gene-transduced and the *ex vivo* cultured Tn cells expressed the anti-MART-1 TCR at equal levels to the Tem and Tcm cells, they demonstrated the lowest potential for effector function *in vitro*, similar to the biological activity of Tn cells *in vivo*.

Molecular signatures of *in vitro* cultured T lymphocytes

To determine the molecular signature of TCR gene-transduced *in vitro* cultured T cells, we performed microarray analysis on purified Tem, Tcm and Tn sub-populations (Figure 5 and supplementary Table 2). Gene expression profiles of these sub-populations exhibited dramatic differences (Figure 5 on left). We amplified specific regions of the large heat map for detailed presentation on the right (C1 to C5). In the C1 region, we focused on the genes more abundantly expressed in Tem followed by Tcm and Tn, and consistent with other reports (16), observed up-regulation of EOMES, TNF, ID2, IFNG, GZMB, GZMH and KLRB1 in Tem cells. In the C2 region, we amplified a list of genes that were highly expressed in Tcm population. Several genes were over-expressed in Tcm including IRX3, GATA3, ITGB1, TNFRSF4 and LILRB3 but the significance of these results remains to be elucidated. In C3, C4 and C5, we showed 3 regions with genes highly expressed on Tn sub-population, and observed several genes previously reported to be associated with Tn cells (CD127/IL7R, STAT1, LEF1 and CCR7). The molecular signature markers CD27, CCR7, CD127/IL7R and CD70 (Figure 2) identified by FACS analysis were confirmed by microarray analysis (Figure 5).

Tcm show enhanced engraftment and persistence in NOD/SCID/ γ c $^{-/-}$ mice

There is a growing evidence that less-differentiated T lymphocytes are more effective *in vivo* in terms of persistence and tumor treatment (7,8,20,30). To compare the *in vivo* engraftment of human T cells in an immunodeficient mouse model, we transduced PBL and expanded these cells for 2 weeks, followed by cell sorting into Tem and Tcm populations defined by CD45RO and CD62L (supplementary figure 1, the number of Tn cells was insufficient for *in vivo* studies). Tem and Tcm were injected into NOD/SCID/ γ c $^{-/-}$ mice (1.7×10^6 cells per mouse) without any exogenous cytokine support. Thirty-seven days post injection, the mice were sacrificed and lymphocytes from spleen, LN and lung were extracted. The Tcm sub-population showed significant engraftment and persistence *in vivo* compared to the Tem sub-population. The anti-tumor TCR transduced by lentiviral vector was also detected in Tcm sub-population (Figure 6). The total number of infused human cells in spleen, LN and lung were calculated and the data indicated that the Tcm had a significantly increased engraftment and persistence compared to Tem in the NOD/SCID/ γ c $^{-/-}$ mouse model (Figure 6).

Discussion

When T lymphocytes encounter foreign antigen, they undergo robust expansion in a short period enabling sufficient numbers of antigen specific T cells to eradicate foreign antigens. The outcome of this immune response usually generates two types of memory T cells, Tem and Tcm, which co-exist with the antigen inexperienced Tn populations (12–15). Tcm and Tem cells can be distinguished phenotypically and functionally. Compared to Tem, Tcm cells express L-Selectin/CD62L and chemokine receptor CCR7, and secrete IL-2 rather than IL-4 and IFN γ . The expression of CD62L is also characteristic of Tcm and Tn cells ability for cell extravasation through high endothelial venules and migration to areas of secondary lymphoid organs (12,13), whereas Tem cells lack CD62L and are resident in tissues such as lung, liver and gut (31). Antigen-specific memory T cells against viruses or other microbes can be found in both Tcm and Tem subsets. Tcm cells have been shown to confer superior protection against viruses (22), bacteria (22) and cancer (20) in several different model systems compared with Tem cells. Recently, a distinct subset of self-renewing human memory CD8 $^{+}$ T cells was isolated from both Tcm and Tem cells (18).

ACT using autologous T lymphocytes genetically modified to express anti-tumor TCRs can mediate tumor regression and this strategy can now be applied to patients with common

epithelial cancers (3,32,33). At present, what is not well understood is how *in vitro* culture and gene transduction affects the T cells' phenotype and function. Interestingly, when we analyzed the genetically modified and *in vitro* expanded T lymphocytes defined by differentiated markers CD45RO and CD62L, we always observed the same distribution of T cells subsets as are observed in vivo cells, i.e., Tem (CD45RO+CD62L-), Tcm (CD45RO+CD62L+) and Tn (CD45RO-CD62L+). The percentage of cells in each sub-population varied with extent of *in vitro* culture, but usually at day 14 the Tcm dominated the populations. In this report, we demonstrate that the majority of these TCR gene-engineered, *in vitro* cultured T cells not only have the phenotype of Tcm cells, but also mimic the biological properties of *in vivo* Tcm cells. A similar phenotypic distribution has been reported using γ -retroviral vector transduction of a chimeric antigen receptor in human T cells(34).

In vitro generated Tcm cells have comparable cytokine and lytic abilities as *in vitro* generated Tem cells, but their survival in NOD/SCID/ γ c-/- mice is significantly enhanced compared to Tem cells. The gene expression pattern by microarray analysis of cultured Tem, Tcm and Tn of CD8 T cells clearly indicated that these three sub-populations of cells were at different differentiation stages (Figure 5). The data are consistent with gene expression patterns known to be higher at Tem including EOMES(35), TNF(36), ID2(37), IFNG(38), GZMB(39), GZMH(40) and GNLY(41), and to be lower at Tn including IL7R(42), STAT1(43), LEF1(44) and CCR7(45). We also amplified a part from the heat map showing up-regulation of genes in Tcm; the possible significance of these genes in defining the role of Tcm is still under investigation. Some of the genes up-regulated in Tcm cells include: IRX3 (46), a member of the Iroquois homeobox gene family which plays a role in an early step of neural development; GATA3 (47), a transcriptional activator, which binds to the enhancer of the T cell receptor alpha and delta genes; ITGB1 (48), a member of the integrin family, which is involved in cell adhesion and recognition in a variety of processes including embryogenesis, hemostasis, tissue repair, immune response and metastatic diffusion of tumor cells; TNFRSF4 (49), a member of the TNF-receptor superfamily that promotes the expression of apoptosis inhibitors BCL2 and BCL2L1/BCL2-XL; and lastly, LILRB3 (50), an immunoglobulin-like receptor that is thought to control inflammatory responses and cytotoxicity to help focus the immune response and limit autoreactivity (Figure 5).

This molecular signature was confirmed at the protein level where Tcm and Tem produced more IFN γ (and less IL-2) compared to Tn. Due to the lack of GZMB and GZMH (Figure 5), Tn cells had less potential to kill target cells compared to Tcm and Tem. Most significantly, when the *in vitro* generated anti-tumor T lymphocytes were sorted into Tem and Tcm sub-populations and infused into NOD/SCID/ γ c-/- mice (51,52), Tcm showed increased engraftment and persistence in spleen, LN and lung compared to that of Tem (Figure 6). Since CD62L acts as a homing receptor, it may have helped Tcm to enter secondary lymphoid tissues and achieved sustained engraftment. Currently, we are investigating the role of CD62L on surface of human T lymphocytes, as it might represent an important molecule that could be modified for adoptive cell therapy.

In this study, we systematically analyzed *in vitro* generated anti-tumor T lymphocytes phenotypically defined by differentiation markers CD45RO and CD62L, and demonstrated that they mimicked the outcome of immune T cells *in vivo* after encountering antigen. The presence of a second T cell receptor introduced by gene transfer does not alter the ability of these T cells to behave as memory cells, which may be essential for their ability to effectively treat cancer. The short-term *in vitro* culture generated anti-tumor T lymphocytes show a dominant Tcm phenotype that are endowed with intrinsic advantages such as *in vivo* persistence, suggesting that Tcm-like cells maybe the most appropriate type of cell for adoptive cell therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

The *in vitro* cultured anti-tumor T lymphocytes exhibit into distinct populations defined by differentiated markers CD45RO and CD62L. **A.** Schematic illustration of *in vitro* generated anti-tumor T lymphocytes. PBMC were activated by anti-CD3/CD28 beads on day 0, and on day 1 post-stimulation the cells were transduced with lentiviral vector harboring MART-1 antigen TCR, and maintained in culture for 14 days. **B.** FACS analysis of *in vitro* cultured T lymphocytes defined by CD45RO and CD62L. The *in vitro* cultured T lymphocytes were gated on CD8 T cells and analyzed using differentiated markers CD45RO and CD62L. The phenotype of the cultured T cells from 3 donors at day 0 and day 14 is shown in dot plots. **C.** The *in vitro* cultured anti-tumor T lymphocytes exhibit distinct populations defined by differentiated markers CD45RO and CD62L. Cells were gated on CD8, analyzed for CD45RO and CD62L expression followed by the individual markers as indicated. The expression of individual markers CD27, CCR7, etc. in each quadrant on left was overlaid on the right. The histograms from red, blue, and green indicate cells from Tem, Tcm and Tn respectively. Tem, effector memory T cells; Tcm, central memory T cells; Tn, naïve T cells. PBMC derived from donor 3.

Figure 2.

Biological activity of the *in vitro* cultured anti-tumor T lymphocytes. **A.** Phenotypical analysis of transduced PBMC cells at day 14. Cells were gated for CD3 and further analyzed using a panel of differentiation markers as denoted on top of each FACS image. The fold expansion within 14 days was shown on right of each donor. **B.** Cytokine induction. Transduced PBMC cells were co-cultured with melanoma lines, and cytokine production of IFN γ and IL-2 was measured by ELISA. **C.** Specific lysis of melanoma lines. The specific lytic activities of transduced CD8 $^{+}$ T cells were determined by co-culture with ^{51}Cr -labeled tumor cells at the indicated ratio and the release of ^{51}Cr was measured. 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. Both melanoma lines express MART-1, 526 is HLA-A2 positive and 938 is HLA-A2 negative.

Figure 3.

Distinct biological activities of *in vitro* generated anti-tumor T cells from sorted Tem, Tcm and Tn populations. **A.** Sorting of differentiated sub-populations of CD8 T cells defined by CD45RO and CD62L. PBMC were activated, transduced with the lentiviral vector harboring anti-tumor TCR, cultured for 14 days post-transduction and CD8 T cells were sorted using CD45RO and CD62L. **B.** Sorted cells were post-analyzed by FACS, and the expression of TCR was evaluated by Tetramer staining. **C.** Distinct biological activities of sorted Tem, Tcm and Tn sub-populations. The sorted sub-populations representing Tn, Tcm and Tem were co-cultured with tumor lines and the secretion of IFN γ and IL2 were determined by ELISA (data are the mean of triplicate cultures). To measure the lytic activity of sorted sub-populations, cells were co-cultured with ^{51}Cr -labeled tumor cells at the indicated ratio and the release of ^{51}Cr was measured. Results representing mean of triplicate cultures were plotted.

Figure 4.

In population shows reduced potential for effector-target formation.

The sorted TCR engineered CD8 sub-populations defined by CD45RO and CD62L were co-cultured with CFSE pre-labeled tumor line 526. At the end of incubation, the cells were stained with CD8 and T cell:tumor cell conjugate visualized by FACS analysis. The lower left panel is a schematic illustration showing the potential interactions of co-cultured cells. The effector-conjugate formation was calculated using conjugated population divided by unconjugated in each sub-population and presented as percentage as shown in the figure on the bottom right. The data represent the mean $\pm$ SD from triplicate co-culture. The percentage of conjugate formation from Tem, Tcm versus Tn showed statistically significant, $p < 0.05$.

Figure 5.

Sorted Tem, Tcm and Tn populations display unique gene expression patterns. The *in vitro* cultured CD8⁺ T lymphocytes, further defined by CD45RO and CD62L, were sorted into Tcm, Tem and Tn populations, and total RNA was extracted for analysis of gene expression profiles by cDNA microarray. Two-way hierarchical clustering of sorted T cell samples was conducted and 2-fold differentially expressed genes were chosen for analysis. A set of 597 probe IDs was identified as differentially expressed among cultured T cell subsets and used for clustering. Each row in the heat map represents a gene and each column represents a T subset sample. As shown in the color bar, red indicates higher than median expression (up-regulation) and blue indicates lower than median expression (down-regulation). The five main gene clusters are indicated on the right from C1 to C5. Higher magnification of clusters is displayed on the right. Gene symbols are shown next to the heat map.

Figure 6.

Tcm sub-population shows an increased engraftment and persistence in NOD/SCID/γc^{-/-} mice. Sorted Tem and Tcm sub-populations were injected intravenously into NOD/SCID/γc^{-/-} mice without administration of IL-2. Thirty-seven days later, mice were sacrificed and T lymphocytes were isolated from spleen, lymph nodes (LN) and lung. Upper panel, purified T lymphocytes were stained with anti-human CD3 or CD3 plus MART-1 Tetramer-PE. Data from one representative mouse is shown in the top panels. Lower panels, for all mice the absolute number of persisting human T lymphocytes was calculated by multiplying the total lymphocyte counts from each organ by the percentage of CD3+CD8+ or CD3+CD4+ cells. The p value reflecting the difference of Tcm and Tem in each organ is shown.